Contribution à l'étude du rôle et du mode d'action de Fsh et de Lh dans le testicule de truite

Elisabeth Sambroni

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THÈSE / UNIVERSITÉ DE RENNES 1
sous le sceau de l'Université Européenne de Bretagne

pour le grade de

DOCTEUR DE L'UNIVERSITÉ DE RENNES 1

Mention : BIOLOGIE

Ecole doctorale (Vie Agro Santé)

présentée par

Elisabeth Sambroni

Préparée à l'unité de recherche INRA UR1037 LPGP
Laboratoire de Physiologie et Génomique des Poissons
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Contribution à l'étude du rôle et du mode d'action de Fsh et de Lh chez la truite

Thèse soutenue à Rennes
le 22 novembre 2013

devant le jury composé de :

Sylvie DUFOUR
Directrice de Recherche MNHN / *Rapporteur*

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Avant-propos

Cette thèse est basée sur les articles qui suivent et qui sont référencés dans l'introduction par les chiffres romains correspondants :

I. Elisabeth Sambroni, Florence Le Gac, Bernard Breton and Jean-Jacques Lareyre. 2007 Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. Journal of Endocrinology 195, 213–228

II. Elisabeth Sambroni, Latifa Abdennebi-Najar, Jean-Jacques Remy, Florence Le Gac. 2009 Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Oncorhynchus mykiss* General and Comparative Endocrinology 164 107–116

III. Sambroni Elisabeth, Rolland Antoine D., Lareyre Jean-Jacques and Le Gac Florence. 2013 Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis. Journal of Molecular Endocrinology 50(1):1-18

IV. Sambroni Elisabeth, Lareyre Jean-Jacques and Le Gac Florence. 2013 Fsh controls gene expression in fish both independently of and through steroid mediation. Plos One 8 (10) e76684.(Sambroni *et al.* 2013b)

Règles adoptées dans le manuscrit pour la nomenclature des protéines et des gènes :

Gènes humains : en italique et lettres majuscules (Ex *IGF1*)
Protéines humaines : en lettres majuscules (Ex IGF1)

Gènes de souris : en italique, avec la première lettre en majuscule (Ex *Igf1*)
Protéines de souris : en lettres majuscules (Ex IGF1)

Gènes de poissons : en italique et en lettres minuscules (Ex *igf1b*)
Protéines de poissons : première lettre en majuscule (Ex Igf1b)

Abréviations

11KT : 11-kétotestostérone
3β-HSD: 3β-hydroxysteroid dehydrogenase/D5-D4 isomerase
ADNc : Acide Désoxyribo Nucléique complémentaire
AMH / amh : Anti-Mullerian Hormone (hormone antimüllérienne)
AMPc: Adenosine Mono Phosphate cyclique
APPL1 : Adaptor Protein containing a PH domain, PTBdomain and Leucine zipper motif 1
DHP ou 17α-20βOHP: 17 alpha,20 beta-dihydroxy-4-pregnen-3-one
E2: Estradiol 17-béta
EDS : Ethylène Diméthane Sulfonate
EGFR: Epidermal Growth Factor Receptor
ERα : Récepteur alpha des estrogènes
ERβ: Récepteur béta des estrogènes
FAO: Food and Agriculture Organization
FSH /Fsh: Follicle Stimulating Hormone
FSHR/Fshr: Follicle Stimulating Hormone Receptor
GnRH: Gonadotropin-Releasing Hormone
GPCR: Récepteur couplé aux protéines G (G-protein coupled receptor)
Gα/s: Sous-unité alpha stimulatrice
HHG: Hypothalamo-Hypophyso-Gonadique
HIS: Hybridation *in situ*
Hpg : Hypogonadique
ICC: Immuno Cyto Chimie
IGFI: Insulin-like Growth Factor 1
KO: Knock-Out
LH/Lh: Luteinizing Hormone
LHCGR/Lhcgr: Luteinizing Hormone/Chorionic Gonadotropin Receptor
LRR: Leucine-Rich Repeats
MT: 17α-methyl testosterone
NCR: N-terminal Cystein Rich
PKA: Protéine Kinase dépendant de l'AMPc
RGS: Rapport Gonado Somatique
RIA: Radio Immuno Assay
T : Testostérone
TGFβ : Transforming Growth Factor béta

SOMMAIRE

INTRODUCTION

Les groupes des poissons (Chondrichtyens, Actinoptérygiens et Sarcoptérygiens) sont les plus représentés parmi les vertébrés et comptent près de 28 000 espèces vivant dans des milieux très différents (salinité, luminosité, température, profondeur). Il n'est donc pas surprenant qu'une multitude de stratégies de reproduction aient été développées pour s'adapter à ces environnements aquatiques très différents. Cette diversité se manifeste dans la sexualité, qui présente une variété de modalités naturelles, allant du gonochorisme à l'hermaphrodisme, dans le comportement de reproduction, dans le comportement parental et dans les caractéristiques de la gamétogenèse, telles que la durée, le taux de fécondité, le rendement spermatogénétique.

Ce travail de thèse ayant pour objet le contrôle hormonal de la spermatogenèse, j'ai volontairement axé l'introduction sur le mâle.

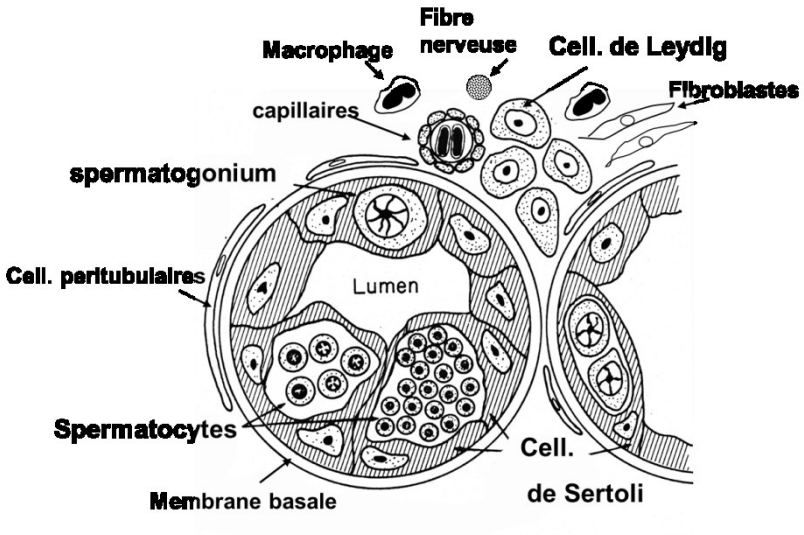
I. La spermatogenèse

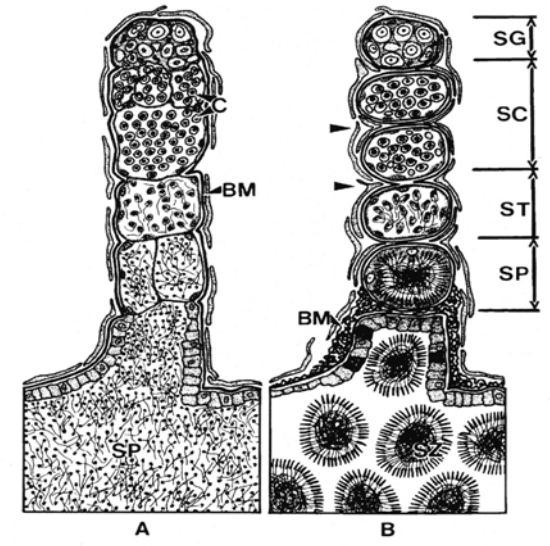
Notre connaissance de la spermatogenèse chez les poissons est limitée à quelques espèces seulement, étudiées soit en tant que modèles pour la recherche fondamentale soit pour répondre à des besoins de l'aquaculture (poisson zèbre, médaka, guppy, saumon, truite, tilapia, anguille, poisson-chat, morue, flétan).

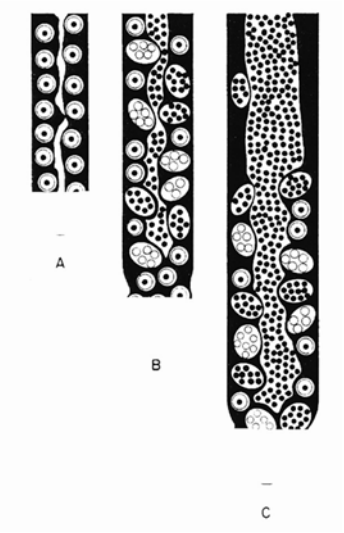
I.1. Structure du testicule

Les testicules sont des organes pairs qui assurent une double fonction gamétogène et endocrine. L'organisation de base du testicule est commune à tous les vertébrés et consiste en un compartiment germinal séparé par une membrane basale du compartiment interstitiel, où l'on trouve le plus souvent les cellules stéroïdogènes de Leydig, les capillaires sanguins, des fibroblastes et des macrophages ainsi que des cellules neurales. Des cellules péritubulaires de type myoïde forment une couche cellulaire parfois incomplète à la surface externe du compartiment germinal.

Au sein du compartiment germinal, l'épithélium germinal des téléostéens est organisé très différemment de celui des mammifères: les cellules de Sertoli et les cellules germinales constituent des cystes clos, qui représentent l'unité fonctionnelle spermatogénétique où les







Type lobulaire à spermatogenèse restreinte

Type tubulaire à spermatogenèse non restreinte

Figure 1 : Organisation spatio-temporelle du compartiment germinal chez le mâle. **A** : Coupe transversale d'un lobule. Les cellules de Sertoli et des cellules germinales au même stade de développement constituent des cystes clos (d'après Billard 1982). **B** : Représentation des 2 types d'arrangements du compartiment germinal (adapté de Grier 1980). A gauche, les cystes émigrent de l'extrémité fermée vers les canaux déférents. A droite, les cellules germinales prolifèrent et se différencient de façon synchrone au sein de cystes, puis les spermatozoïdes sont libérés dans la lumière du tube. SG : spermatogonies, SC : spermatocytes, ST : spermatides, SP : spermatozoïdes.

cellules germinales, issues d'un spermatogonium primaire, évoluent de façon synchrone. Des cystes à différents stades peuvent cependant se côtoyer au sein d'un tubule (Fig. 1A). De ce fait (et à la différence des mammifères), il semble que chez les téléostéens les cellules de Sertoli à un temps t, n'entourent et ne sont en contact qu'avec un clone de cellules germinales au même stade de différenciation. Au fil de la maturation, les cystes, dont la taille s'accroît considérablement, contiennent successivement des nombres croissants de spermatogonies, spermatocytes, spermatides et enfin s'ouvrent pour libérer des spermatides tardives ou les spermatozoïdes dans la lumière du tubule. Tandis que chez les amniotes le testicule adulte contient un nombre fixe de Sertoli qui supporte les vagues successives de spermatogenèse, chez les poissons ces cellules conservent leur capacité à proliférer pour constituer et accompagner la croissance des nouveaux cystes, comme par exemple au début de la recrudescence chez les espèces saisonnières. La barrière hémato-testiculaire est surtout constituée par la jonction entre cellules de Sertoli à la périphérie des cystes (plutôt qu'à la périphérie des tubules) et se mettrait en place plus tardivement que chez les mammifères, après la méiose (Schulz *et al.* 2010). Chez certains ordres de téléostéens les cellules de Sertoli peuvent présenter une activité phagocytique très intense afin de résorber les corps résiduels, et surtout les nombreux spermatozoïdes non excrétés après la période de frai.

Se surimposant à l'arrangement en cystes de l'épithélium, deux grands types d'organisation spatiotemporelle du compartiment germinal sont observés (Grier *et al.* 1980) Le premier type, dit restreint, est caractérisé par des lobules avec une terminaison distale fermée, et l'autre extrémité ouverte sur les canaux efférents. Dans ce cas, les cystes à spermatogonies sont restreints à la terminaison distale, puis migrent le long du lobule vers les canaux excrétoires au fur et à mesure de la différenciation germinale (Fig. 1B). Ce type d'arrangement est retrouvé chez les téléostéens d'ordres supérieurs tels que les Athériniformes, les Cyprinodontiformes (ex : guppy, gambusie) et les Béloniformes (ex : médaka) (Parenti *et al.* 2004). Le second type, dit non restreint, présente un réseau de tubules, souvent connectés entre eux ou ouverts à chaque extrémité sur les canaux excrétoires. Dans ce cas, des spermatogonies souches sont présentes tout au long des tubules, et les cystes maturant migrent peu et de façon centripète vers la (future) lumière du tubule.

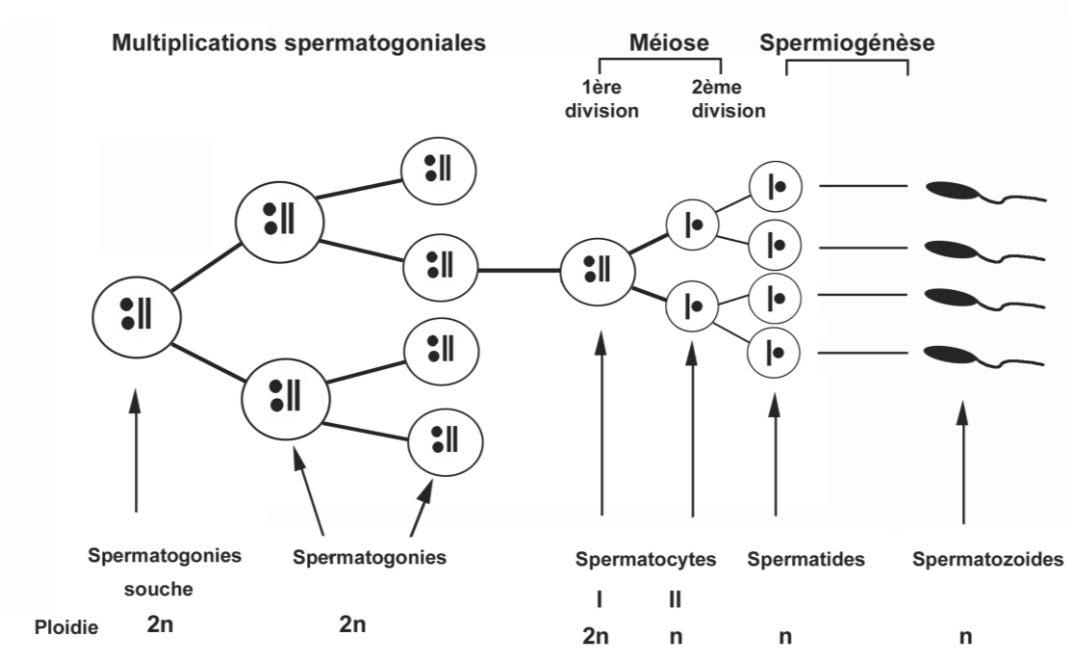


Figure 2 : Représentation schématique des 3 principales étapes de la spermatogenèse.

I.2. Le déroulement de la spermatogenèse

La fonction gamétogène ou spermatogenèse est un processus long et complexe qui aboutit à la production d'un très grand nombre de gamètes, les spermatozoïdes, aptes à féconder un ovule.

Chez toutes les espèces, la spermatogenèse se déroule en trois temps: i) une première phase de multiplication des spermatogonies par des divisions cellulaires mitotiques, ii) une phase de divisions méiotiques qui transforment les cellules diploïdes en cellules haploïdes et iii) une phase de modifications morphologiques, appelée spermiogenèse, qui transforme une spermatide ronde en un spermatozoïde porteur d'un flagelle.

La plupart des téléostéens présentent un rythme annuel de reproduction, parfois très marqué chez les espèces des zones tempérées à froides. Ainsi, certaines espèces constituent des modèles d'intérêt pour des études portant sur les régulations endocrines de la gamétogenèse car on peut s'adresser à des stades précis de maturation de la gonade et avoir accès à des populations de cellules germinales synchrones et représentatives d'un stade de différenciation.

a. Cycle reproducteur chez notre modèle d'étude, la truite arc-en-ciel

La truite arc-en-ciel (*Oncorhynchus mykiss*) est un salmonidé originaire d'Amérique du Nord. C'est une espèce à reproduction saisonnière, capable de se reproduire plusieurs fois au cours de sa vie d'adulte. Un cycle de reproduction, sous nos latitudes et dans les conditions naturelles, dure normalement 1 an. Pour la souche automnale, la plupart des mâles sont sexuellement immatures au cours de leur première année et la gamétogenèse active démarre en février-mars de la seconde année. Ils commencent à émettre du sperme à partir du mois de septembre et 100 % des mâles excrètent du sperme au mois de novembre. La période de spermiation s'étend sur environ 5 mois. Le développement des spermatogonies souches en spermatozoïdes se produit de façon répétée au cours des cycles de reproduction successifs.

La spermatogenèse active (Figure 2) se caractérise d'une part, par la prolifération et la différenciation des cellules somatiques de Sertoli et d'autre part, par la prolifération rapide et la différenciation des spermatogonies A. Cette phase détermine le rendement final de la spermatogenèse. Lors de l'initiation de la spermatogenèse, une cellule de Sertoli entoure une

	Cellules germinales						
Stades	Gonies A	Gonies B	Spermatocytes	Spermatides	Spermatozoïdes	Spermiation	RGS (%)
I	**+++**						**0.06 ± 0.003**
II	**+++**	**+**					**0.08 ± 0.003**
IIIa	**+++**	**++**	**+**				
IIIb	**++**	**+++**	**++**	**(+)**			**0.10 ± 0.007**
IV	**+**	**++**	**+++**	**++**	**(+)**		**0.3 ± 0.03**
V	**(+)**	**+**	**++**	**+++**	**++**		**1.3 ± 0.1**
VI	**(+)**		**+**	**++**	**+++**		**4.3 ± 0.2**
VII	**(+)**		**(+)**	**(+)**	**++++**	**(+)**	**5.5 ± 0.3**
VIII	**(+)**				**++++**	**+ à +++**	**3.8 ± 0.2**

Note. + à ++++ indique l'abondance relative de chaque type de cellules germinales observé durant l'analyse histologique. (+) = rare

Tableau 1 : Définition chez la truite arc-en-ciel des stades de maturation gonadique en fonction de la présence des différents types de cellules germinales et de leur représentativité dans les cystes. Le RGS moyen mesuré à chaque stade est indiqué dans la dernière colonne du tableau (Adapté de Gomez et al., 1999).

cellule germinale pour former un cyste dans lequel la spermatogonie va subir six cycles de division mitotiques successives pour générer un grand nombre de spermatogonies B (au maximum 64 par cyste). Les spermatogonies B qui sont diploïdes sont à l'origine des spermatocytes primaires qui doublent leur matériel génétique et initient la première division méiotique pour former des spermatocytes secondaires. Les spermatocytes secondaires subissent la seconde division méiotique qui aboutit à la réduction chromosomique pour former les cellules haploïdes appelées spermatides. La différenciation des spermatides en spermatozoïdes, ou spermiogénèse, se traduit par une forte condensation de la chromatine associée à une pause dans l'activité transcriptionnelle du génome, une organisation particulière des mitochondries et la formation du flagelle nécessaire à la motilité des gamètes. Chez la truite, comme chez la plupart des téléostéens, les spermatozoïdes n'ont pas d'acrosome. A la fin de la spermiogénèse la paroi sertolienne des cystes devenue de plus en plus mince s'ouvre et les spermatozoïdes sont libérés dans la lumière du tubule, c'est la spermiation (Pudney 1995). Elle est accompagnée d'une hydratation des gonades et du sperme (Legendre *et al.* 1988). La période de spermiation peut se poursuivre plusieurs mois mais la qualité du sperme diminue fortement dans le temps du fait de phénomènes de vieillissement des spermatozoïdes (Billard 1979).

La spermatogenèse n'est pas totalement synchrone au sein de la gonade car elle débute dans les cystes situés dans les régions les plus internes et antérieures puis elle se développe progressivement vers les cystes les plus externes et postérieurs qui maturent les derniers.

Huit stades de développement de la gonade ont été définis en fonction de la présence des différents types de cellules germinales et de leur représentativité parmi les cystes (Voir tableau 1 adapté de (Gomez *et al.* 1999).

Au cours d'un cycle de reproduction, le testicule présente une grande variation de son poids et le rapport gonadosomatique (RGS), égal au poids des gonades divisé par le poids corporel et exprimé en pourcentage, passe de 0.06 % à 5-7 % juste avant la spermiation. Ce caractère reflète la forte cyclicité et l'importance des proliférations spermatogoniales mais témoigne aussi d'un fort rendement du processus dû à un faible taux d'apoptose (~30 à 40 % ; (Vilela *et al.* 2003). Cette caractéristique est particulièrement d'intérêt chez les espèces à fécondation externe pour augmenter la probabilité de rencontre entre un spermatozoïde et un ovule dans le milieu extérieur et garantir la survie de l'espèce.

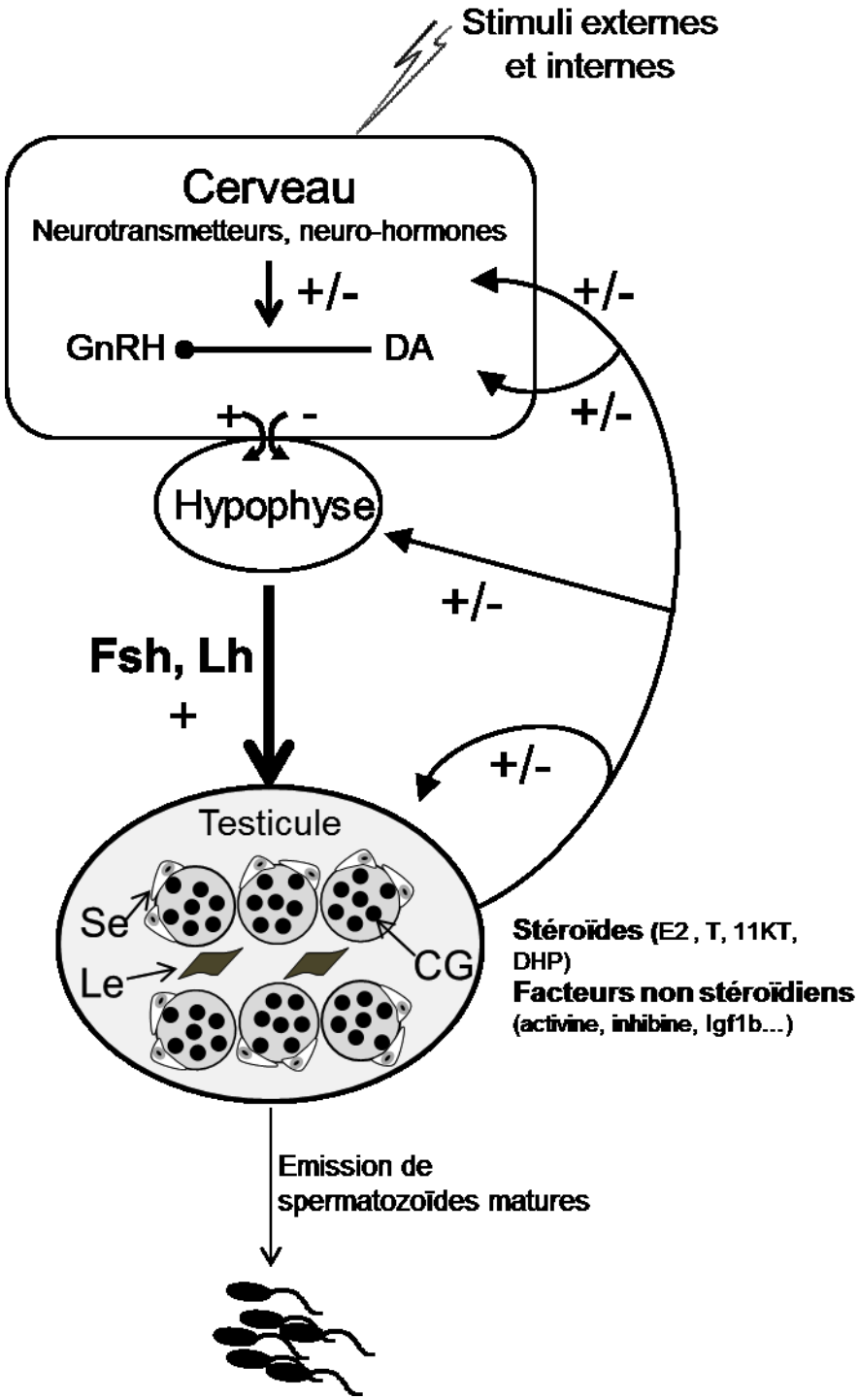


Figure 3 : L'axe hypothalamo hypophyso gonadique chez les téléostéens mâles. DA dopamine, Se, cellules de Sertoli, Le : cellules de Leydig, CG : cellules germinales (Adapté de Weltzien et al., 2004).

II. Le contrôle hormonal de la spermatogenèse

Chez tous les vertébrés, la spermatogenèse est régulée par les interactions endocriniennes entre l'hypophyse et les cellules gonadiques. Ce système endocrinien, désigné sous le nom d'axe hypothalamo-hypophyso-gonadique (HHG), englobe une série de mécanismes de signalisation qui coordonnent la spermatogenèse et la stéroïdogenèse (Fig. 3).

A. Les gonadotropines

Chez les mammifères, le rôle de substances produites par l'hypophyse antérieure sur la croissance et le cycle de reproduction chez la femelle a été démontré dès les années 1920 (Evans *et al.* 1922; Hewitt 1929). Plus tard, Fevold et ses collègues (Fevold *et al.* 1931) réussirent à séparer deux fractions à partir d'hypophyses de brebis, l'une stimulant la croissance folliculaire et l'autre stimulant la lutéinisation. Ces deux fractions furent donc désignées respectivement Follicle Stimulating Hormone (FSH) et Luteinizing Hormone (LH). Une dizaine d'années plus tard, l'implication des hormones hypophysaires dans le contrôle de la reproduction des téléostéens a été mise en évidence par des expériences d'hypophysectomie réalisées chez différentes espèces de poissons (Pickford *et al.* 1957; Billard *et al.* 1969; Hoar 1969). Chez le mâle, l'hypophysectomie conduit à la suppression de la spermatogenèse (Hoar 1965) et l'injection d'extraits hypophysaires permet de régénérer complètement la spermatogenèse (Yamazaki *et al.* 1968) (Billard *et al.* 1970).

1. Structure des gonadotropines

Chez les poissons, deux gonadotropines hypophysaires ont été isolées et caractérisées à partir de 1988 (Suzuki *et al.* 1988a; Swanson *et al.* 1991; Van Der Kraak *et al.* 1992; Govoroun *et al.* 1997). Initialement dénommées GTH I et GTH II, elles sont structurellement assimilables à FSH et LH, respectivement et portent désormais le même nom. Les gonadotropines appartiennent à la famille des glycoprotéines hypophysaires qui inclut aussi la TSH. Ces trois hormones sont constituées de 2 sous-unités α et β liées entre elles par des liaisons non covalentes. La sous-unité α porte la spécificité zoologique tandis que les sous-unités béta différentes pour chacune des hormones leur confèrent leur spécificité d'action biologique (Pierce *et al.* 1981). Alors que chez les mammifères il n'y a qu'une seule sous-unité alpha, commune aux 3 glycoprotéines hypophysaires pour une même espèce, chez plusieurs espèces de poissons deux sous-unités alpha ont été décrites. Chez le saumon chum (*Oncorhynchus keta*), Fshβ peut se lier à chacune des 2 sous-unités alpha, alors que Lhβ se lie

principalement à α2 (Suzuki *et al.* 1988a). Au contraire, chez la truite arc-en-ciel (*Oncorhynchus mykiss*) les 2 sous-unités α1 et α2 participent à la formation des dimères Fsh et Lh (Govoroun *et al.* 1997).

L'alignement des sous-unités α de poissons et de mammifères révèle une forte similarité, allant de 60 à 90 %. En particulier, la position des dix cystéines et des deux sites putatifs de N-glycosylation est conservée (Querat *et al.* 1990). La similarité de séquence entre Fsh beta et Lh beta se situe autour de 30 % tandis que les pourcentages d'identité avec les sous-unités beta humaines sont de 40 % et 48 %, respectivement (Sekine *et al.* 1989; Govoroun *et al.* 1997).

Chaque hormone possède des chaînes glucidiques diverses par les sucres qui les composent, leur longueur et le nombre de ramifications. La variabilité des chaînes glucidiques influence la biosynthèse, la sécrétion, la clairance métabolique et l'activité biologique des hormones (Pierce *et al.* 1981).

2. Régulation de la synthèse et de la sécrétion des gonadotropines

Chez les mammifères, les gonadotropines sont synthétisées dans l'hypophyse antérieure, au sein des mêmes cellules. Les cellules gonadotropes sont caractérisées par la présence de grains de sécrétion de 2 types, les uns petits (100 à 300 nm) et denses aux électrons et les autres plus gros (jusqu'à 600 nm) et moins dense aux électrons.

Contrairement à ce qui est observé chez les mammifères, la Fsh et la Lh sont synthétisées dans des cellules différentes de l'hypophyse tout au long du cycle de reproduction (Nozaki *et al.* 1990a; Nozaki *et al.* 1990b; Kagawa *et al.* 1998). Chez le saumon coho, seules les cellules sécrétant Fsh sont présentes dans l'hypophyse avant la puberté et leur nombre augmente au cours de la spermatogenèse et de la vitellogenèse. Les cellules à Lh n'apparaissent qu'à l'initiation de la spermatogenèse et augmentent fortement au moment de la maturation finale (Nozaki *et al.* 1990a). L'autre particularité des poissons par rapport aux mammifères est l'absence de système porte, l'hypophyse étant irriguée indépendamment du cerveau. En conséquence, les facteurs hypothalamiques hypophysiotropes sont délivrés aux cellules hypophysaires directement à partir des neurones sécréteurs.

Chez les téléostéens comme chez tous les vertébrés, la GnRH, décapeptide produit dans le cerveau, stimule la production et la sécrétion des gonadotropines par l'hypophyse (Breton *et al.* 1993; Lin *et al.* 1996; Kumakura *et al.* 2004). Trois formes de GnRH ont été identifiées

et coexistent dans le cerveau de la plupart des poissons téléostéens, y compris les salmonidés primitifs (Vickers *et al.* 2004). Cependant, à ce jour, seules les formes GnRH2 et GnRH3 ont été décrites chez la truite (Kah *et al.* 2007). D'autres régulateurs neuroendocriniens tels que la dopamine, l'acide gamma aminobutirique (GABA), la norépinephrine, la sérotonine et le neuropeptide Y participent aussi au contrôle de la sécrétion des gonadotropines (Breton *et al.* 1991; Mananos *et al.* 1999). La stimulation des gonades par les gonadotropines se traduit par la production de facteurs gonadiques tels que l'inhibine, l'activine et les stéroïdes sexuels (E2, T) qui vont exercer en retour des rétro-actions positives ou négatives sur la production et la libération des gonadotropines (Breton *et al.* 1996; Saligaut *et al.* 1998; Dickey *et al.* 1998), soit par des effets directs sur les cellules hypophysaires gonadotropes, soit indirectement via le système à GnRH dans le cerveau (Zohar *et al.* 2010).

3. Profils hormonaux au cours du cycle

Chez le mâle comme chez la femelle des espèces très cycliques, la Fsh et la Lh présentent des évolutions très significatives en termes de transcrit et protéine hypophysaires et en termes de concentration circulant dans le plasma sanguin. Les premières données sur les évolutions des contenus hypophysaires et des taux plasmatiques de la Fsh et de la Lh ont été obtenues chez les salmonidés, pour lesquels des dosages radioimmunologiques avaient été mis au point suite à la purification des 2 gonadotropines (Suzuki *et al.* 1988c; Prat *et al.* 1996; Govoroun *et al.* 1998). Chez le saumon et la truite, la Fsh est la seule gonadotropine détectable dans la circulation sanguine en début de cycle. Ses niveaux plasmatiques augmentent au cours de la phase d'accroissement entre ovocyte primaire et ovocyte secondaire chez la femelle, et au stade de prolifération des spermatogonies chez le mâle, et s'élèvent à nouveau au cours de la période péri ovulatoire et avant la spermiation (Prat *et al.* 1996; Gomez *et al.* 1999). Au contraire, la Lh n'est pas détectable au début du cycle de reproduction mais augmente fortement aux étapes finales de la gamétogenèse à savoir la maturation ovocytaire ovulation et la spermiation (Prat *et al.* 1996; Breton *et al.* 1998).

A défaut de disposer d'un dosage spécifique de chacune des gonadotropines, c'est l'expression des gènes qui a été étudiée pour avoir une information sur les variations liées à des stades de développement gonadique, même si les niveaux d'ARNm ne reflètent pas nécessairement les niveaux circulants des hormones. Chez l'anguille, Fshβ est exprimée chez l'immature alors que l'expression de Lhβ n'apparaît que chez les mâles spermiants et les

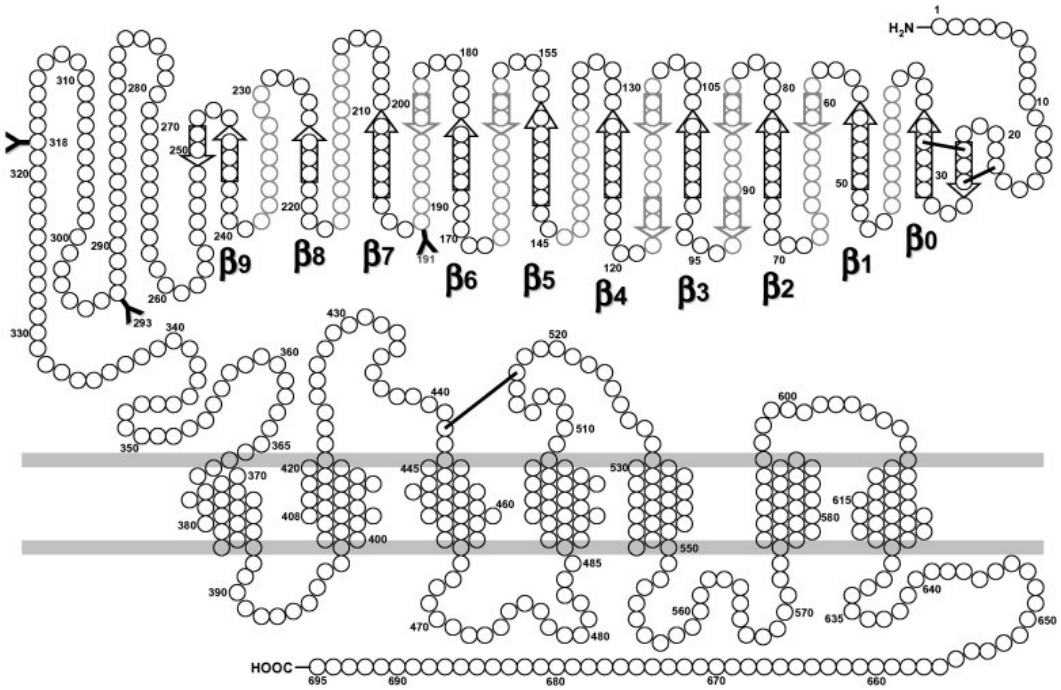


Figure 4 : Représentation schématique de la structure secondaire typique d'un récepteur d'une glycoprotéine. Sont représentés le domaine extracellulaire N-terminal, les 7 hélices du domaine transmembranaire connectées entre elles par 3 boucles extracellulaires et 3 boucles intracellulaires, et le domaine intracellulaire COOH-terminal. Les feuillets β de chaque LRR sont indiqués par des flèches. Les sites porentiels de N-glycosylation sont indiqués par « Y ». Le trait noir indique la présence d'un pont disulfure entre les résidus cystéine conservés présents dans les boucles extracellulaires 1 et 2 (D'après Levavi-Sivan *et al*., 2010).

femelles ovulées (Yoshiura *et al.* 1999). Chez les salmonidés mâles, le transcrit *fshb* est généralement faiblement exprimé chez l'immature et ses niveaux s'élèvent en début de spermatogénèse. Ils restent élevés et stables pendant la spermatogénèse et diminuent légèrement (saumon atlantique) ou restent élevés (truite) au moment de la spermiation (Weil *et al.* 1995; Gomez *et al.* 1999; Maugars *et al.* 2008). Au contraire, les niveaux de *lhb* augmentent modérément au cours de la spermatogénèse et très fortement à la spermiation (Weil *et al.* 1995; Gomez *et al.* 1999; Maugars *et al.* 2008). Chez d'autres espèces, les 2 transcrits fluctuent en parallèle au cours du cycle de reproduction. Par exemple chez le flétan, l'expression des 2 transcrits *fshb* et de *lhb* est détectée chez le juvénile et augmente fortement chez les mâles spermiants (Weltzien *et al.* 2004). De même, chez japanese flounder, *fshb* et *lhb* sont exprimés à des niveaux élevés dès les premières étapes de la spermatogenèse et augmentent régulièrement jusqu'à la spermiation (Kajimura *et al.* 2001).

4. Mécanisme d'action des hormones gonadotropes

Comme chez les autres vertébrés, une fois sécrétées dans la circulation sanguine, les gonadotropines se lient à des récepteurs membranaires exprimés sur les cellules somatiques des gonades. La liaison de Lh (GTH II) à des récepteurs de haute affinité a été démontrée pour la première fois dans l'ovaire de salmonidés (Salmon *et al.* 1984; Breton *et al.* 1986; Kanamori *et al.* 1987) et dans le testicule de truite (Le Gac *et al.* 1987). L'existence de deux activités de liaison distinctes a ensuite été démontrée par des études de liaison de la Fsh (GTH I) et de la Lh (GTH II) sur des ovaires et des testicules de saumon (Yan *et al.* 1992). La présence de 2 récepteurs distincts a été confirmée par le clonage moléculaire de 2 ADNc différents chez plusieurs espèces de poissons: saumon (Oba *et al.* 1999a; Oba *et al.* 1999b; Maugars *et al.* 2006), poisson-chat (Bogerd *et al.* 2001; Kumar *et al.* 2001b; Kumar *et al.* 2001c; Vischer *et al.* 2003a) et poisson zèbre (Kwok *et al.* 2005; So *et al.* 2005).

a. La structure des récepteurs

Ces récepteurs membranaires appartiennent à la super famille des récepteurs couplés aux protéines G (GPCR) et plus particulièrement, au sous-groupe des récepteurs des hormones glycoprotéiques (Figure 4). Ils sont caractérisés par 3 domaines fonctionnels comprenant :

- un long domaine extracellulaire en N-terminal qui contient 9 à 10 motifs répétés riches en leucine (LRRs) d'environ 25 acides aminés, critiques pour la spécificité de liaison avec le ligand (Salesse *et al.* 1996; Couture *et al.* 1996; Levavi-Sivan *et al.* 2010)

- Un domaine d'ancrage à la membrane composé de 7 motifs transmembranaires

- Un domaine intracellulaire qui possède plusieurs résidus serine, thréonine et tyrosine qui sont des sites potentiels de phosphorylation par des kinases intracellulaires. Ce domaine interagit avec le complexe protéine Gs - adénylate cyclase.

Si la structure générale des récepteurs des gonadotropines est conservée chez tous les vertébrés, le domaine extracellulaire présente des différences avec celui des récepteurs mammaliens. En particulier, les sites potentiels de N-glycosylation diffèrent en nombre et en séquences entre les poissons et les mammifères. De plus, dans la séquence de Fshr, on ne retrouve pas la région N-terminale riche en cystéines (NCR) typique (Kumar *et al.* 2001a).

b. Profil d'expression des récepteurs au cours du cycle

L'évolution du nombre de récepteurs à la gonadotropine maturante (GTH II), au cours d'un cycle de reproduction, a été étudiée chez la truite par des études de liaison classiques, avant que la seconde gonadotropine n'ait été purifiée et que les séquences codantes des récepteurs ne soient connues. Chez le mâle, le nombre de récepteurs est faible dans les testicules en régression, augmente pendant la spermatogenèse et atteint un maximum au moment de la spermiation (Le Gac *et al.* 1987; Le Gac *et al.* 1988). Chez la femelle, le nombre de récepteurs augmente significativement en début de vitellogenèse, reste constant et plus faible pendant la vitellogenèse et est multiplié par 10 dans l'ovaire post-ovulation (Breton *et al.* 1989).

L'accès aux séquences codantes des 2 récepteurs chez des espèces de poisson de plus en plus nombreuses a rendu possible et plus aisée l'analyse temporelle de l'expression des récepteurs au cours d'un cycle de reproduction. Chez la barbue de rivière femelle, l'expression de Lhcgr est détectée à un faible niveau, relativement stable tout au long du cycle et présente un pic au moment la maturation ovocytaire finale et de l'ovulation (Kumar *et al.* 2001c). Inversement, l'expression de Fshr diminue au moment de l'ovulation et augmente pendant la phase de recrudescence ovarienne (Kumar *et al.* 2001b). Chez la femelle tilapia,

espèce à pontes multiples, les niveaux des transcrits *fshr* sont élevés au cours de la vitellogenèse précoce, diminuent en fin de vitellogenèse et s'élèvent à nouveau après la maturation. Les transcrits *lhcgr* quant à eux sont faiblement exprimés au cours de la vitellogenèse précoce et augmentent progressivement pendant la vitellogenèse pour atteindre leur plus haut niveau au cours de la maturation ovocytaire (Oba *et al.* 2001). En dépit d'une différence de mode de reproduction de ces 2 espèces, les profils d'expression des récepteurs sont similaires et suggèrent une implication de Fshr dans la phase de recrudescence ovarienne, tandis que Lhcgr jouerait un rôle dans la maturation ovocytaire finale et l'ovulation (Kumar *et al.* 2001a). Cependant, chez la truite, Bobe et collaborateurs ont montré que l'acquisition de la compétence folliculaire (à maturer) est associée à une expression accrue de Fshr mais pas de Lhcgr (Bobe *et al.* 2003).

Un groupe japonais a suivi l'évolution des transcrits *fshr* et *lhcgr* chez 3 espèces de téléostéens, le saumon amago, le tilapia et le chabot (*Pseudoblennius cottoides*). Globalement, les deux récepteurs présentent des profils d'expression semblables chez les mâles avec un pic pendant la spermiation. Chez les femelles, *fshr* est fortement exprimé au cours de la vitellogenèse, tandis que *lhcgr* présente une forte expression uniquement au cours de la maturation finale (Hirai *et al.* 2002).

Chez la truite mâle, l'abondance des transcrits *fshr* fluctue au cours du cycle et reste plutôt faible. Une première augmentation de leur expression se produit au cours de la phase de forte activité méiotique. L'expression de *lhcgr* augmente progressivement au cours du processus spermatogénétique et présente un pic au cours de la période de spermiation (Kusakabe *et al.* 2006).

Même si la mesure de l'abondance des transcrits *fshr* et *lhcgr* ne permet pas à elle seule de conclure définitivement sur le rôle des récepteurs dans la gamétogenèse, les données accumulées vont dans le sens d'une implication de Fshr dans les étapes précoces du cycle et de Lhcgr dans les étapes de maturation finale et d'émission des gamètes.

c. Localisation atypique des récepteurs chez les poissons

Chez les mammifères, les récepteurs de la FSH sont exprimés sur les cellules de Sertoli dans le testicule et sur les cellules de la granulosa dans l'ovaire (Simoni *et al.* 1997). Les récepteurs de la LH sont exprimés sur les cellules de Leydig dans le testicule. Dans l'ovaire,

ils sont situés sur les cellules de la thèque interne à partir du stade préantral et apparaissent sur les cellules de la granulosa du follicule pré ovulatoire en milieu de phase folliculaire. Les premiers travaux pour localiser les récepteurs des gonadotropines chez les poissons ont fait appel à des essais de liaison des ligands sur coupes de tissu gonadique de saumon, suivie d'autoradiographie (Miwa *et al.* 1994). Les auteurs ont ainsi pu localiser un récepteur de type I (Fshr) à la fois sur les cellules de la thèque et de la granulosa, tandis que le récepteur de type II (Lhcgr) n'a été identifié que sur les cellules de la granulosa des follicules pré ovulatoires. Chez le mâle, les récepteurs de type I (Fshr) sont clairement présents sur les cellules bordant les tubules, probablement les cellules de Sertoli, chez le juvénile, l'adulte immature et l'adulte mature. Déjà, la présence des récepteurs de la Fsh sur les cellules de Leydig était envisagée par les auteurs du fait de la détection de sites de liaison sur le tissu conjonctif, tissu qui ne contient pas de cellules de Sertoli. La présence des récepteurs des gonadotropines de type II (Lhcgr) n'a été clairement détectée que sur les cellules interstitielles (cellules de Leydig) chez les mâles pré spermiants, à partir du stade V.

Avec le développement des outils moléculaires et le clonage des récepteurs chez plusieurs espèces de poissons, la localisation cellulaire des transcrits ou des protéines a été recherchée par hybridation *in situ* (HIS) ou par immunocytochimie (ICC), respectivement. La présence de Fshr a ainsi été décrite dans le compartiment tubulaire mais aussi sur les cellules de Leydig chez plusieurs poissons comme l'anguille, le poisson-chat africain, le poisson zèbre et une espèce de mérou (Ohta *et al.* 2007; Garcia-Lopez *et al.* 2009; Alam *et al.* 2010; Garcia-Lopez *et al.* 2010). Chez la sole sénégalaise, alors qu'une première publication faisait état d'une expression spécifique de chacun des récepteurs, *fshr* dans les cellules de Sertoli et *lhcgr* dans les cellules de Leydig (Chauvigne *et al.* 2010), le même groupe, sur la base de travaux plus récents faisant appel à des approches complémentaires d'HIS et d'ICC, a conclu à la présence de récepteurs Fshr à la fois sur les cellules de Sertoli et sur les cellules de Leydig, tandis que le récepteur Lhcgr est exprimé uniquement sur les cellules de Leydig (Chauvigne *et al.* 2012).

Récepteur	Rein	Rein antérieur	Rate	Vésicule séminale	Cerveau	Cœur	Branchies	Foie	Espèce	Références
Fshr	-	NT	NT	NT	-	NT	NT	-	Saumon Amago	(Oba *et al.* 1999a)
	-	-	Oui	-	-	NT	-	-	Channel catfish	(Kumar *et al.* 2001b)
	-	-	NT	Oui	-	NT	NT	-	Poisson-chat	(Bogerd *et al.* 2001)
	Oui	NT	NT	NT	-	-	-		Poisson zèbre	(Kwok *et al.* 2005)
	-	-	NT	NT	-	-	Oui	-	Saumon atlantique	(Maugars *et al.* 2006)
	-	-	-	-	-	-	-	-	Bar	(Rocha *et al.* 2007)
Lhcgr	-	NT	NT	NT	Oui	NT	NT	-	Saumon Amago	(Oba *et al.* 1999b)
	Oui	NT	-	NT	-	NT	Oui	-	Channel catfish	(Kumar *et al.* 2001c)
	-	Oui	NT	-	Oui	Oui	NT	-	Poisson-chat	(Vischer *et al.* 2003a)
	-	NT	NT	NT	-	NT	-	Oui	Poisson zèbre	(Kwok *et al.* 2005)
					Oui	Oui	Oui	Oui	Saumon atlantique	(Maugars *et al.* 2006)
	Oui	Oui	Oui	NT	Oui	Oui	Oui	Oui	Bar	(Rocha *et al.* 2007)

Tableau 2 : Données de l'expression extragonadique des transcrits des récepteur de Fsh et de Lh chez différentes espèces de poissons. NT : non testé

d. Expression extragonadique des récepteurs des gonadotropines

L'expression des récepteurs Fshr et Lhcgr est prédominante dans les gonades mais selon les espèces on détecte aussi une expression modérée à forte des transcrits correspondants dans d'autres tissus (Voir tableau 2) incluant le plus fréquemment le rein (Gautier *et al.* 2011) ou le rein antérieur mais aussi la branchie, le cerveau, l'hypophyse et le cœur. A noter que chez le poisson-chat africain l'expression de *fshr* est plus forte dans la vésicule séminale que dans le testicule (Bogerd *et al.* 2001) tandis que l'expression de *lhcgr* est de même intensité dans le testicule et dans le rein antérieur (Vischer *et al.* 2003a). On ignore si les récepteurs extragonadiques sont fonctionnels et quel pourrait être leur rôle. Chez les mammifères, FSHR et LHCGR sont aussi exprimés dans des tissus autres que les gonades. Par exemple, FSHR est présent à la surface des ostéoclastes et des cellules souches mésenchymateuses. La FSH a été impliquée dans la régulation de la masse osseuse (Sun *et al.* 2006). Une expression de FSHR est décrite dans les cellules endothéliales des vaisseaux sanguins de la gonade mais aussi dans les vaisseaux sanguins irriguant de nombreuses tumeurs. Cela suggère un lien entre l'expression de FSHR et l'angiogenèse (Radu *et al.* 2010; Siraj *et al.* 2013). Chez l'humain, la présence de LHCGR a été rapportée dans un grand nombre de tissus incluant la thyroïde (Frazier *et al.* 1990), le cerveau, l'utérus, le placenta, l'os, la prostate, l'épididyme, les vésicules séminales (Rao *et al.* 2007).

e. Sélectivité de liaison des récepteurs

Bien que la structure générale en 3 grands domaines fonctionnels des récepteurs de poissons soit similaire à celle de tous les autres vertébrés, y compris les reptiles, les oiseaux et les mammifères, plusieurs études chez des téléostéens indiquent que les sélectivités de liaison des récepteurs aux gonadotropines de poissons sont moins strictes vis-à-vis de leur ligand par rapport à celles observées chez les mammifères. Chez les poissons, des études de liaison avec des gonadotropines hypophysaires ou recombinantes purifiées ont montré des liaisons croisées. Chez le saumon coho, le récepteur de type I (Fshr) se lie préférentiellement à la Fsh mais aussi à la Lh même si l'affinité reste plus faible (Yan *et al.* 1992). Chez la carpe, les récepteurs purifiés des hormones gonadotropes se lient préférentiellement à leur ligand respectif, mais ils reconnaissent aussi modérément l'autre hormone (Basu *et al.* 2002). Des études fonctionnelles utilisant des lignées cellulaires mammaliennes exprimant les récepteurs ont montré que, selon l'espèce, l'un ou l'autre des récepteurs pouvait être activé par les 2

hormones. Chez le poisson-chat africain, les hormones recombinantes cfFsh et cfLh activent Fshr avec une potentialité similaire (Vischer *et al.* 2003b), alors que chez le saumon amago, seule la Fsh est capable d'activer Fshr (Oba *et al.* 1999a). Cependant, la sélectivité hormonale semble aussi dépendre de l'origine et la nature des gonadotropines. Chez le poisson zèbre, la FSH bovine active les 2 récepteurs Fshr et Lhcgr, tandis que la LH bovine active spécifiquement Lhcgr (Kwok *et al.* 2005). Les hormones recombinantes de poisson zèbre se comportent à l'inverse vis-à-vis de leurs récepteurs, c'est-à-dire que la Fsh active seulement Fshr, et la Lh active les 2 récepteurs (So *et al.* 2005).

Il apparaît difficile de conclure sur la sélectivité réelle ou non des récepteurs aux gonadotropines de poissons dans un contexte tissulaire car la plupart des analyses décrites ci-dessus ont été réalisées dans un contexte cellulaire hétérologue avec bien souvent l'emploi de gonadotropines hétérologues ou d'hormones recombinantes.

f. Transduction du signal

En se liant à leurs récepteurs, les gonadotropines déclenchent une cascade de réactions en chaîne qui commence généralement par l'activation de la protéine Gαs. Une fois activée, la protéine Gαs va interagir avec l'effecteur majeur de la voie de signalisation, l'adénylate cyclase pour produire le second messager l'AMPc (Zhang *et al.* 1991). Cette voie de signalisation canonique Gαs/cAMP/protéine kinase A (PKA) a longtemps été considérée comme le mécanisme de signalisation clé mis en jeu par l'activation de FSHR (Means *et al.* 1974; Dattatreyamurty *et al.* 1987). Cependant de nombreux travaux montrent que plusieurs autres voies sont activées et se connectent en réseau. Elles incluent principalement les autres mécanismes de transduction couplés aux autres sous-unités Gα, la voie de signalisation dépendante des β-arrestines, la transactivation de EGFR et la signalisation induite par APPL1 (Gloaguen *et al.* 2011). Chez les poissons, il existe peu de données sur les voies de transduction autres que celle de l'AMPc. Chez le poisson-chat africain, l'autre voie marginale identifiée est la phospholipase C et l'inositol triphosphophate (Vischer *et al.* 2003a).

5. Fonctions biologiques des gonadotropines

Chez les mammifères, plusieurs modèles ont été exploités ou développés pour étudier le rôle physiologique des gonadotropines : mutations pathologiques chez l'homme, la souris *hpg* hypogonadique à cause d'une mutation de la GnRH qui entraîne une absence de gonadotropines hypophysaires et circulantes, l'invalidation des gènes *Fshb*, *Lhb*, *Fshr* ou *Lhcgr* chez la souris, l'immunisation passive ou active contre FSH, LH, FSHR ou LHCGR.

a. Phénotypes observés par invalidation génique de FSH et de son récepteur

Le développement de souris mutantes dépourvues des gonadotropines ou de leurs récepteurs respectifs a conduit à une meilleure compréhension de la biologie de ces hormones (Kumar 2005; Kumar 2007). Les phénotypes observés en absence d'une hormone ou de son récepteur spécifique sont quasiment identiques, ce qui suggère l'absence d'interférence *in vivo* avec d'autres couples ligand-récepteur structurellement similaires. Cependant le phénotype observé chez les souris mâles dans le cas du KO du récepteur de FSH est plus sévère que celui observé dans le KO de FSHβ, le nombre de cellules de Leydig ainsi que les taux plasmatiques de testostérone étant plus faibles dans le premier cas (Kumar 2005; Huhtaniemi 2006). Ce phénomène peut s'expliquer par l'existence d'une activité constitutive des récepteurs de FSH qui pourrait être suffisante pour maintenir une certaine fonctionnalité de FSHR dans les cellules de Sertoli, indépendamment de la présence du ligand FSH (Baker *et al.* 2003). Chez le mâle, FSH ne semble pas indispensable à la fertilité de l'adulte. Cependant en son absence, on constate un déséquilibre hormonal accompagné d'un retard de la puberté et d'une baisse du rendement spermatogénétique liée à une taille des testicules et un volume des tubes séminifères réduits. De plus en absence de FSHβ ou de son récepteur, on observe une baisse de la motilité du sperme et d'autres défauts partiels dans la qualité des spermatozoïdes (forme de la tête, condensation de la chromatine). A noter que le phénotype observé chez les femelles KO pour FSHR ou pour FSHβ est plus sévère puisqu'il se traduit par l'infertilité.

Chez les mammifères, l'un des rôles de la FSH dans la régulation de la spermatogenèse est la stimulation de la prolifération des cellules de Sertoli qui est limitée à la période périnatale. Les cellules de Sertoli sont les seules cellules somatiques en contact direct avec les cellules germinales. FSH module la production de facteurs produits par les cellules de Sertoli

qui vont agir sur le développement et le fonctionnement des cellules de Leydig et des cellules germinales. Les cellules de Sertoli fournissent le soutien physique et nutritionnel nécessaire à la spermatogenèse qui se déroule dans l'espace intercellulaire entre les cellules de Sertoli. Il est bien admis que le nombre de cellules de Sertoli conditionne le nombre de cellules germinales produites, et en conséquence, la taille des testicules. Dans le testicule immature de plusieurs espèces mammaliennes y compris les primates, FSH stimule aussi la production de cellules de Leydig (Kerr *et al.* 1985).

b. Phénotypes observés par invalidation génique de LH et de son récepteur

L'absence de LHβ chez des souris mâles se traduit par l'hypogonadisme, le blocage de la différenciation des cellules de Leydig, une forte diminution des niveaux de testostérone plasmatique et intratesticulaire et l'hypoplasie des glandes sexuelles accessoires (Ma *et al.* 2004). La spermatogenèse et les fonctions sertoliennes sont touchées : il y a absence totale de spermatides allongées et certains marqueurs sertoliens (*Inhba*, *Inhbb* et *Amh*) apparaissent régulés de façon aberrante (Ma *et al.* 2004).

La seule fonction indispensable de LH au sein du testicule adulte semble être de réguler la synthèse de testostérone. Le traitement de souris KO pour LHCGR par de la testostérone exogène, permet de complètement restaurer la spermatogénèse. Le traitement de souris *hpg*, déficientes en FSH et LH, par de la testostérone permet aussi une récupération qualitative de la spermatogénèse. En absence de remplacement de la testostérone, la spermatogenèse s'arrête à la phase de méiose chez les souris *hpg*. L'effet positif de la testostérone ne dépend pas des autres facteurs produits par les cellules de Leydig, puisque la T seule restaure totalement la spermatogenèse chez des rats traités à l'éthylène diméthane sulfonate (EDS), cytotoxine spécifique des cellules de Leydig (Holdcraft *et al.* 2004).

c. Gènes FSH et LH dépendants chez les mammifères

Au cours de la dernière décennie, ce sont les mécanismes moléculaires mis en jeu dans l'action de la FSH et des androgènes qui ont fait l'objet d'une attention particulière. Grâce au développement des technologies à haut débit permettant l'étude exhaustive du transcriptome, de nombreux gènes exprimés dans les gonades mâles et régulés par FSH et/ou par les androgènes ont pu être identifiés *in vitro* (McLean *et al.* 2002) et *in vivo* (Sadate-Ngatchou *et al.* 2004; Meachem *et al.* 2005). Bien que ces études génèrent des listes de gènes qui se

recoupent faiblement, il ressort que FSH module l'expression de gènes exprimés dans les cellules de Sertoli mais aussi, indirectement, dans les cellules de Leydig et dans les cellules germinales. Ces gènes sont impliqués, entre autres, dans la régulation du cycle cellulaire, dans la transduction du signal ou encore dans l'architecture de l'épithélium séminifère.

d. Fsh et Lh ont-elles des rôles et mécanismes d'action définis chez les poissons ?

Chez les espèces à reproduction saisonnière, chaque gonadotropine présente un profil de sécrétion spécifique suggérant que Fsh et Lh ont des fonctions différentes. Les activités biologiques des hormones natives purifiées ou des hormones recombinantes ont été le plus souvent évaluées dans des tests *in vitro* sur la stéroïdogenèse : stimulation de la production de stéroïdes ou stimulation de l'expression de gènes codant des enzymes de la stéroïdogenèse. Il existe peu d'études sur les effets des hormones sur la gamétogenèse précoce ou tardive.

- *Fsh*

Chez les poissons, une des actions importantes de la Fsh sur les fonctions gonadiques est la stimulation de la stéroïdogénèse, chez le juvénile (Swanson *et al.* 1989) comme chez l'adulte (Planas *et al.* 1995). Fsh a également des effets sur la spermatogénèse. Chez le poisson-chat africain, Fsh simule la prolifération des cellules de Sertoli (Schulz *et al.* 2003), et chez la truite, Fsh stimule *in vitro* la prolifération des spermatogonies A. Cet effet nécessite la médiation des cellules de Sertoli (Loir 1999). L'initiation de la spermatogénèse (prolifération des spermatogonies et début de méiose) est associée, au moins chez les salmonidés à une augmentation des taux plasmatiques de Fsh (Suzuki *et al.* 1988c; Gomez *et al.* 1999). Chez l'anguille, la Fsh recombinante (rec-Fsh) induit à la fois le renouvellement des spermatogonies et l'entrée en spermatogenèse. Après 30 jours de traitement, tous les stades de développement des cellules germinales sont présents. La spermatogenèse induite par rec-Fsh est inhibée en présence d'un inhibiteur de l'enzyme 3β-HSD, le trilostane. Par contre, le trilostane n'inhibe pas la spermatogenèse induite par la 11KT ; cela suggère que l'action de la Fsh sur la spermatogenèse s'exerce via la production d'androgènes (Ohta *et al.* 2007). Cet effet sur la stéroïdogenèse passe probablement par la stimulation de l'expression de gènes de la stéroïdogenèse (Kazeto *et al.* 2008).

In vivo, injectée à des anguilles immatures, la Fsh recombinante augmente de façon significative le poids testiculaire, stimule la production de 11KT et induit l'entrée en spermatogenèse active (Kamei *et al.* 2006).

Chez la femelle, Fsh est le principal régulateur de la croissance et de la maturation folliculaire et Fsh, mais pas Lh, stimule l'incorporation de la vitellogénine dans les ovocytes (Tyler *et al.* 1991; Tyler *et al.* 1997). De plus, Fsh stimule la production d'E2 par des ovocytes en vitellogenèse, en stimulant à la fois l'expression et l'activité de l'aromatase (Montserrat *et al.* 2004).

- *Lh*

Etant donné que l'expression de *lhb* et/ou les taux plasmatiques de Lh ne sont généralement détectés qu'en fin de cycle, on attribue à Lh un rôle dans le contrôle des étapes finales de la gamétogénèse : maturation ovocytaire et ovulation, chez la femelle et spermiogénèse et spermiation, chez le mâle (Prat *et al.* 1996). Chez la femelle, il a été montré, que seule Lh, et pas Fsh, induit la rupture de la vésicule germinative et favorise l'acquisition de la compétence ovocytaire (Kagawa *et al.* 1998; Planas *et al.* 2000). De plus, Lh se révèle plus efficace que la Fsh pour stimuler *in vitro* la production de DHP par des ovocytes pré ovulatoires et par des testicules d'animaux en spermiation (Suzuki *et al.* 1988b; Planas *et al.* 1995).

Chez le brochet, divers extraits gonadotropes hypophysaires injectés à des mâles en fin de spermatogénèse, augmente fortement la quantité de spermatozoïdes émis (Billard *et al.* 1983). La maturation (volume, hydratation) et l'excrétion des spermatozoïdes est sous le contrôle de la Lh, par l'intermédiaire de la DHP. Ces processus ont lieu alors que les niveaux circulants de Lh et de DHP sont les plus élevés.

B. Les stéroïdes sexuels

Trois classes de stéroïdes sexuels, les œstrogènes, les androgènes et les progestagènes sont principalement produites par les gonades mâles (Voir figure 4). Chez les poissons téléostéens, la biosynthèse des stéroïdes suit principalement la voie delta 4 (Fostier *et al.* 1987). Les stéroïdes sexuels présentent des variations importantes de leur taux plasmatique en fonction de l'état de maturation de la gonade et sont les médiateurs principaux de l'action des gonadotropines.

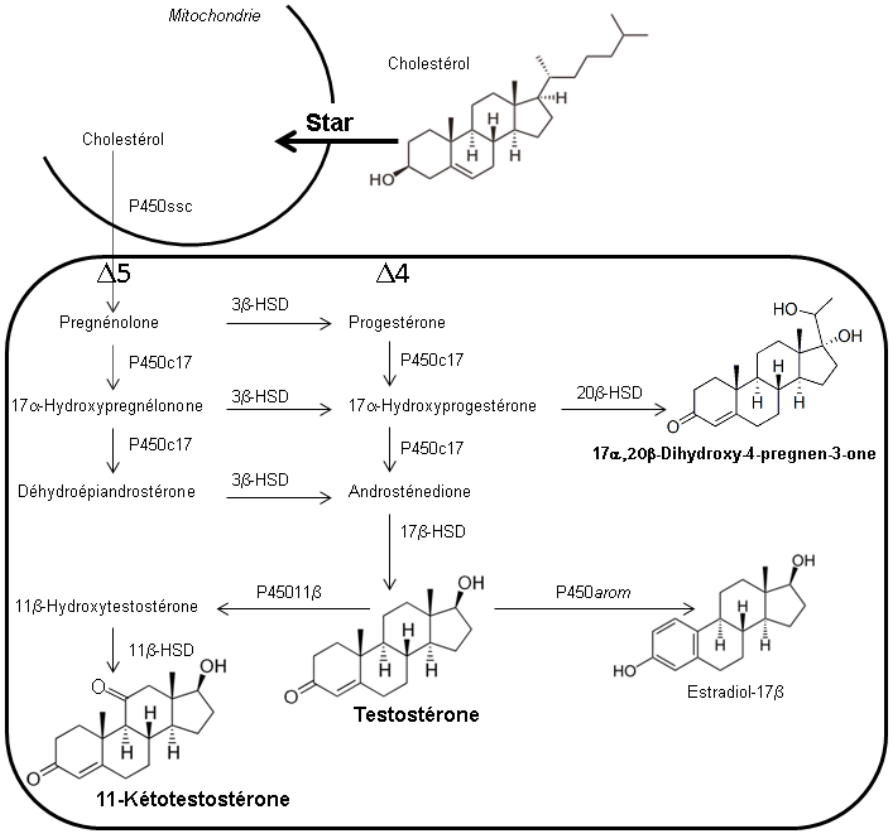


Figure 4 : Schéma de la biosynthèse des stéroïdes dans la gonade de poissons (Adapté de Borg, 1994).

1. Les œstrogènes

Bien que généralement associés au cycle de reproduction de la femelle, les œstrogènes, et en particulier l'estradiol-17β (E2), sont présents chez le mâle à de faibles concentrations dans le plasma. Les œstrogènes sont produits à partir des androgènes par l'aromatase, enzyme qui est exprimée dans toutes les cellules testiculaires (somatiques et germinales) excepté les cellules péritubulaires (Carreau *et al.* 2012). Chez les mammifères, les œstrogènes se lient à 2 types de récepteurs nucléaires, ERα et ERβ, issus de 2 gènes différents. En plus de leur rétroaction négative sur le cerveau, les œstrogènes sont reconnus comme des régulateur paracrines potentiels de la spermatogenèse chez de nombreuses espèces y compris l'homme. Différents modèles animaux ont permis de démontrer l'importance des œstrogènes dans la spermatogenèse (O'Donnell *et al.* 2001). Les souris mâles déficientes en aromatase développent une spermatogenèse anormale, caractérisée par un blocage de la maturation des cellules germinales au stade spermatide. L'invalidation des gènes des récepteurs aux œstrogènes chez la souris a démontré que les mâles ERαKO et ERβKO sont infertiles. L'infertilité des souris adultes ERαKO était liée à l'absence de réabsorption du fluide par les cellules épithéliales des canaux efférents qui induit alors une augmentation progressive de la pression dans le testicule, conduisant à l'atrophie des tubes séminifères et provoquant la destruction des cellules germinales.

Chez les poissons, la gonade mâle est un site majeur de l'expression de 3 formes de récepteurs nucléaires ERα, ERβ1 et ERβ2. L'expression des récepteurs aux œstrogènes a été rapportée dans les cellules somatiques et dans les cellules germinales haploïdes (Wu *et al.* 2001; Menuet *et al.* 2002). Chez l'anguille japonaise, E2 a été associé à l'autorenouvèlement des spermatogonies (Miura *et al.* 1999) tandis que chez la daurade, E2 pourrait être impliqué dans la régression des testicules (Chaves-Pozo *et al.* 2007). Chez la truite, on observe une élévation transitoire de E2 au début du cycle de reproduction corrélée au développement des cellules germinales pré méiotiques (Gomez *et al.* 1999). L'apport exogène d'E2, à des doses physiologiques, chez des truites pré pubères (stades I-II où seules des spermatogonies sont présentes) modifie l'expression de gènes importants pour la stéroïdogenèse (*star*, *hsd3b1*, *cyp19a1* et *cyp19b1*) et pour la spermatogenèse (*amh*, *dmrt1*, *rabp1*) (Données non publiées).

2. Les androgènes

Chez les téléostéens, la spermatogénèse se déroule en présence de niveaux élevés d'androgènes. Il est généralement admis qu'à côté de la testostérone, l'androgène le plus actif

est la 11-kétotestostérone, (11KT) qui est un androgène 11-oxygéné non aromatisable (Borg 1994). Le niveau de testostérone est généralement élevé au cours de la spermatogénèse et atteint un maximum au cours de la spermiogénèse, alors que la 11KT et les autres 11-oxo-androgènes augmentent avec le déroulement de la spermatogénèse et présentent un pic juste avant ou au début de la spermiation (Fostier *et al.* 1982; Mylonas *et al.* 1997; Utoh *et al.* 2004). Les androgènes ont la capacité de soutenir soit le processus entier soit certaines étapes de la spermatogénèse telles que la prolifération des spermatogonies, la formation des spermatocytes (Billard *et al.* 1982; Nagahama 1994). Chez l'anguille immature, la 11KT induit à elle seule la spermatogenèse complète *in vitro*, depuis la prolifération des spermatogonies jusqu'à la production de sperme (Miura *et al.* 1991).

Comme chez les vertébrés supérieurs, les androgènes jouent un rôle dans la maturation de l'hypophyse et dans la régulation de l'expression des gènes des gonadotropines et de la GnRH (Breton *et al.* 1997; Dickey *et al.* 1998; Soga *et al.* 1998). Ils participent à l'établissement des caractères sexuels secondaires et au comportement de reproduction (Borg 1994; Dunlap *et al.* 1998).

3. Les progestagènes

Les progestagènes sont reconnus pour être des stéroïdes importants dans la reproduction des Vertébrés. Chez les mammifères, la progestérone joue un rôle important chez les femelles, en particulier au cours de la gestation.

Chez les téléostéens, la progestine majeure n'est pas la progestérone comme chez les tétrapodes, mais la 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (DHP) ou, chez les espèces marines, la 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S). Chez la femelle, il a été montré que les progestagènes et leurs récepteurs régulent la reprise de la méiose et la maturation des ovocytes (Nagahama 1997; Zhu *et al.* 2003).

Chez les salmonidés mâles, on observe deux pics de sécrétion de DHP au cours du cycle de reproduction : un premier grand pic au moment de la spermiation et un autre moins important au cours de la phase de prolifération des spermatogonies. Chez le mâle, la DHP est reconnue pour jouer un rôle dans les étapes finales de la spermatogénèse.

Elle avance et induit la spermiation chez les salmonidés et les cyprinidés (Ueda *et al.* 1985), augmente la production de sperme (Baynes *et al.* 1985) et stimule la motilité des spermatozoïdes (Miura *et al.* 1992). Plus récemment, la DHP a aussi été impliquée dans les premiers stades de la spermatogenèse. Chez l'anguille japonaise, la DHP est essentielle pour induire la méiose (Miura *et al*. 2006).

En conclusion, chez les poissons, les 3 classes de stéroïdes sexuels, œstrogènes, androgènes et progestagènes, sont d'importants régulateurs dans le processus de spermatogénèse, depuis l'autorenouvèlement des spermatogonies souches jusqu'à la maturation et l'excrétion du sperme.

CONTEXTE ET OBJECTIFS DE LA THESE

I. Contexte finalisé

La compréhension du processus de la spermatogenèse et de son contrôle chez les téléostéens présente un intérêt fondamental en biologie comparative, mais aussi un intérêt pour l'aquaculture. Selon le rapport 2012 de la FAO sur la pêche et l'aquaculture, au cours des trois dernières décennies (1980-2010) la production mondiale de poisson d'élevage destiné à la consommation a été multipliée par près de 12, avec un taux de croissance annuel moyen de 8,8 pour cent. Cela représente la plus forte progression dans le domaine des productions animales. L'aquaculture s'étend dans tous les continents, en termes de nouvelles surfaces et de nouvelles espèces et, pour répondre aux besoins des consommateurs, elle intensifie sa production et diversifie la gamme d'espèces et de types de produits qu'elle fournit.

Pour accompagner ces évolutions et être en mesure de proposer des réponses innovantes aux attentes et besoins des filières de production, il est indispensable d'acquérir les connaissances scientifiques sur les grandes fonctions biologiques qui sont impliquées dans les principaux points de blocage du cycle d'élevage des espèces.

Concernant la fonction de reproduction, les acquis scientifiques doivent permettre de répondre à trois types de demandes liées à la pratique piscicole : la maîtrise de la maturation sexuelle, l'obtention de gamètes en quantité et qualité compatibles avec une production commerciale et les exigences liées à la sélection génétique, le désaisonnement à volonté de la période de ponte pour assurer une fourniture régulière d'alevins. L'entrée en puberté pose des problèmes préjudiciables dans la gestion des cheptels en pisciculture (Taranger *et al.* 2010). Par exemple, chez certaines espèces comme l'esturgeon, le thon, il faut attendre plusieurs années pour atteindre la maturité sexuelle. Inversement chez de nombreuses autres espèces comme les salmonidés, la carpe ou la morue, on observe chez le mâle, un phénomène de maturation sexuelle précoce qui affecte négativement la croissance et la qualité de la chair. Ces deux exemples soulignent l'intérêt de pouvoir intervenir sur la puberté pour l'accélérer ou la retarder selon l'espèce que l'on souhaite élever. Différentes méthodes pour contrôler la

puberté ont été développées en pisciculture comme la sélection génétique, la photopériode, la triploïdisation pour stériliser les poissons ou encore la génération de populations monosexes pour tenir compte d'un caractère avantageux présentant un dimorphisme sexuel. Cependant ces méthodes ne sont pas toujours d'une efficacité satisfaisante et leur transfert vers d'autres espèces n'est pas forcément réalisable. De plus, certaines de ces méthodes peuvent avoir des conséquences négatives pour l'environnement ou poser des problèmes d'acceptabilité par les consommateurs. L'approfondissement des connaissances sur les mécanismes qui contrôlent les phases clés du cycle de reproduction est nécessaire pour pouvoir proposer des méthodes alternatives. Dans cet objectif, les recherches conduites par notre groupe portent sur les régulations endocrines de la puberté dans la perspective de maîtriser l'initiation de la maturation sexuelle des animaux en élevage et sur les régulations endocrines, cellulaires et moléculaires de la spermatogenèse dans la perspective de contrôler la fertilité et la production quantitative et qualitative de la laitance chez les poissons.

II. Problématique et questions scientifiques

Comme rappelé dans l'introduction, chez les mammifères, les activités biologiques des 2 gonadotropines dans la régulation des fonctions gonadiques sont bien délimitées et établies. Chez les poissons, en revanche, les particularismes soulignés dans l'introduction laissent penser que le périmètre d'action des gonadotropines est plus large et, en l'état actuel des connaissances, le rôle respectif de chacune d'elles dans la régulation de la gamétogenèse reste encore à préciser. Trois stratégies complémentaires ont été poursuivies au cours de la thèse pour aborder la question spécifique des rôles respectifs des gonadotropines, avec un intérêt plus particulier pour Fsh au cours de la période pubertaire.

III. Objectifs, choix méthodologiques et grandes lignes de la thèse

III.1 Caractériser les récepteurs des gonadotropines aux plans moléculaire et fonctionnel

L'action des hormones gonadotropes au sein des tissus cibles passe nécessairement par la liaison à des récepteurs membranaires dont la localisation cellulaire, le nombre, la

spécificité de liaison ou encore la désensibilisation vont conditionner la spécificité et l'amplitude de la réponse biologique et moduler la sensibilité hormonale. Une manière d'aborder la question du rôle respectif de chacune des gonadotropines, chez la truite, a donc consisté à s'intéresser aux caractéristiques moléculaires et fonctionnelles des récepteurs homologues de LHCGR et FSHR (**I**). En effet, quand nous avons initié ce travail, les travaux menés chez les poissons décrivaient une spécificité non stricte des récepteurs, pas toujours retrouvée selon les espèces et s'appuyant souvent sur l'utilisation de gonadotropines hétérologues (pisciaires ou mammaliennes). Les données jusqu'alors acquises, rendaient difficile l'établissement d'un schéma généralisable à tous les téléostéens mais aussi compliquaient la compréhension du rôle de chaque gonadotropine. La caractérisation des récepteurs chez le modèle truite devait permettre d'enrichir nos connaissances dans ce domaine. C'est pourquoi nous avons entrepris chez la truite le clonage des séquences codantes des 2 récepteurs.

Leur expression transitoire en cellules mammaliennes nous a permis d'explorer leur spécificité *in vitro* avec l'avantage de disposer d'hormones gonadotropes de truite purifiées.

La connaissance des séquences nous a aussi permis de mesurer de façon fiable le niveau d'expression des transcrits des récepteurs, et l'accès à différents stades de développement au cours du cycle de reproduction, a permis de suivre leur évolution chez le mâle comme chez la femelle.

III.2 La fonction des récepteurs a aussi été abordée *in vivo* dans des expériences d'immunisation active (**II**).

Bloquer l'action des gonadotropines en empêchant leur liaison à leurs récepteurs est une façon originale de questionner leur rôle physiologique. De plus, cette approche peut être envisagée pour inhiber la puberté et le développement des gonades. Afin d'évaluer l'impact d'un tel blocage *in vivo*, nous avons appliqué, pour la première fois chez un poisson, une méthode d'immunisation active contre les récepteurs de la Fsh et de la Lh (Remy et al, 1996). Cette méthode s'était révélée efficace chez les mammifères pour induire des perturbations de la reproduction (retard de la maturation sexuelle et du pic pubertaire de testostérone) chez différents modèles pré pubères ou adultes (Abdennebi et al, 2003 ; Rao et al, 2004). A notre connaissance aucune étude similaire n'avait été menée chez les poissons au moment où les travaux de thèse ont débuté. Or chez ces espèces, l'initiation de la gamétogenèse passe aussi par la sécrétion et l'action des gonadotropines via leur liaison à leurs récepteurs et on a fait

l'hypothèse que l'approche expérimentée chez les mammifères devait être efficace chez la truite. Nous l'avons testée à deux stades du développement sexuel i) chez l'animal pré pubère, avant tout signe de démarrage de la spermatogenèse et ii) au cours de la gamétogenèse. Nous avons évalué les conséquences de l'inactivation des signaux Fsh/Fshr et Lh/Lhcgr sur le développement des gonades (RGS, histologie) et sur les niveaux circulants de stéroïdes sexuels (testostérone, 11-kétotestostérone et œstradiol).

III.3 **Identifier des voies de régulation en jeu dans l'action des gonadotropines sur les fonctions testiculaires chez la truite** (**III** et **IV**)

L'activation du système de transduction par la liaison des gonadotropines à leurs récepteurs peut se traduire par différents modes d'action cellulaire, soit à travers les activités biologiques de protéines phosphorylées, soit par la modification du taux de transcription des gènes. S'intéresser aux effets moléculaires de la Fsh et de la Lh permet d'envisager le rôle respectif des gonadotropines sur leur tissu cible. Connaître les gènes régulés par la Fsh et la Lh permet aussi de repérer des voies de régulation importantes dans le contrôle de la spermatogenèse dans lesquelles ces gènes seraient impliqués. La troisième approche retenue dans ce travail de thèse a donc consisté à identifier des gènes dont l'expression testiculaire est régulée par Fsh et Lh, *in vitro*. Cette analyse a été réalisée *in vitro* car elle permet de s'affranchir de toutes les régulations endocrines existantes à l'échelle de l'individu, de comparer les effets de Fsh et de Lh sur les mêmes tissus (ou pools de tissus) et, qui plus est, elle nécessite de moindres quantités d'hormones hautement purifiées, compatibles avec celles dont nous disposions au laboratoire. Nous avons réalisé des cultures d'explants à partir de gonades pré pubères ou aux toutes premières étapes de maturation (stades I à III) afin de découvrir des voies d'action des gonadotropines lors de la mise en place de la première maturation chez la truite. Nous avons étudié les variations du transcriptome testiculaire à l'aide de puces à ADNc, ce qui a permis ne pas se limiter à quelques candidats mais au contraire d'aborder la question sans *a priori*. En comparaison des technologies actuelles (lames de verre Agilent 44K ou 60K), ces puces contenaient un nombre limité (9024) de sondes ADN complémentaires mais présentaient l'avantage d'être annotées de façon fiable et abondante, notamment en termes de connaissances antérieurement acquises sur leur profil d'expression au cours de la spermatogenèse et en réponse aux stéroïdes sexuels, *in vivo*. Cette annotation particulièrement riche constituait un apport précieux dans l'interprétation de nos données transcriptomiques sur leur signification physiologique.

Les questions spécifiques abordées dans cette partie de thèse sont :

- Les 2 gonadotropines, Fsh et Lh, régulent-elles les mêmes gènes ? (**III**)
- Peut-on repérer des voies de régulation contrôlées par Fsh et Lh qui seraient pertinentes dans le contrôle de la spermatogenèse ? (**III**)
- Les effets de Fsh sur le transcriptome passent-ils principalement par les stéroïdes sexuels ? (**IV**)

Résultats - partie 1

Caractérisation et rôle des récepteurs des hormones gonadotropes chez la truite

Caractérisation moléculaire et fonctionnelle des récepteurs des hormones gonadotropes

Depuis la fin des années 80, le clonage moléculaire des récepteurs des hormones gonadotropes chez les mammifères a ouvert un nouveau champ immense d'exploration de leurs fonctions biologiques et a permis d'étudier leur structure, leur régulation ainsi que les mécanismes de transduction du signal (McFarland *et al.* 1989; Loosfelt *et al.* 1989; Minegishi *et al.* 1990; Sprengel *et al.* 1990; Minegishi *et al.* 1991; Gudermann *et al.* 1992; Remy *et al.* 1995).

Une dizaine d'années plus tard, les séquences codantes des récepteurs Fshr et Lhcgr furent publiées pour la première fois chez un poisson, le saumon amago (*Oncorhynchus rhodurus)* (Oba *et al.* 1999a; Oba *et al.* 1999b). Au commencement des travaux de thèse, les séquences des récepteurs étaient disponibles pour quelques téléostéens appartenant à des familles différentes: le poisson-chat africain, *Clarias gariepinus* (Bogerd *et al.* 2001; Vischer *et al.* 2003a), la barbue de rivière, *Ictalurus punctatus* (Kumar *et al.* 2001b; Kumar *et al.* 2001c), le poisson zèbre, *Danio rerio* (Laan *et al.* 2002; Kwok *et al.* 2005) et le saumon atlantique, *Salmo salar* (Maugars *et al.* 2006). Les études conduites *in vitro* pour tester la sélectivité des récepteurs vis-à-vis des 2 ligands Fsh et Lh décrivaient, selon les espèces et les hormones utilisées dans les tests, une faible sélectivité relative de l'un ou l'autre des récepteurs. Cette observation compliquait la compréhension du rôle biologique des récepteurs et par là même, celui des gonadotropines. Par exemple, l'existence d'une interaction croisée entre la Fsh et le récepteur de Lh pouvait expliquer la forte potentialité de la Fsh à stimuler la stéroïdogenèse.

Le clonage des récepteurs, leur surexpression dans un système cellulaire pour des tests de fonctionnalité et de sélectivité ainsi que l'étude temporelle de leur expression au cours du cycle de reproduction pouvaient apporter une contribution utile à la compréhension des fonctions biologiques des récepteurs et plus largement du rôle des gonadotropines dans la fonction de reproduction chez un nouveau modèle d'intérêt agronomique, la truite.

Le clonage de deux ADNc différents a permis de montrer qu'il existe 2 récepteurs distincts, apparentés aux récepteurs de Fsh et de Lh mammaliens et pisciaires. La structure secondaire générale est conservée avec les 3 domaines caractéristiques des récepteurs couplés aux protéines G comprenant un grand domaine extracellulaire N-terminal de liaison du ligand, un domaine d'ancrage à la membrane avec 7 domaines transmembranaires, et un domaine intracellulaire en C-terminal responsable de l'interaction avec les protéines G.

Cependant, nous avons constaté que certains déterminants structurels du domaine N-terminal étaient différents chez les récepteurs pisciaires par rapport aux récepteurs des espèces mammaliennes. Ces modifications incluent un motif riche en leucine supplémentaire sur le récepteur Fsh en lieu et place d'un pont disulfure. Les conséquences éventuelles de ces modifications structurelles sur la sélectivité des récepteurs restent encore à déterminer.

Les 2 récepteurs sont exprimés chez les individus juvéniles et à tous les stades de la gamétogenèse mâle et femelle et l'expression de leurs transcrits augmente fortement en fin de cycle (maturation ovocytaire et ovulation, spermiation) en accord avec les études de liaison réalisées chez d'autres salmonidés.

La sélectivité des récepteurs a été étudiée *in vitro* en surexprimant de manière indépendante les récepteurs dans une lignée cellulaire hétérologue (COS-7) et en les activant avec l'une ou l'autre des gonadotropines hypophysaires purifiées. Sur la base de ces études de fonctionnalité *in vitro*, nous concluons que les récepteurs Fshr et Lhcgr de truite sont efficacement et préférentiellement activés par leur ligand respectif bien que des réponses croisées de faible amplitude aient été observées avec les doses d'hormones les plus fortes.

Ce travail a fait l'objet d'un premier article, publié dans Journal of Endocrinology et intitulé « Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line ». Ces résultats ont aussi été présentés sous forme d'une communication au congrès « 8th International Symposium on Reproductive Physiology of Fish » (ISRPF) qui s'est tenu à St Malo en juin 2007.

Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line

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Abstract

In vertebrates, gonadotropins (GTHs) (FSH and LH) are two circulating pituitary glycoprotein hormones that play a major role in the regulation of gonadal functions, including gonadal cell proliferation/differentiation and steroidogenesis. In mammals, it is well known that their biological effects are mediated by highly specific membrane-bound receptors expressed preferentially on the somatic cells of the gonads. However, in fish, binding and functional studies have shown that cross-reactivity may occur in GTH receptors depending on the species. To understand the molecular mechanisms involved in GTH actions, functional characterization of trout GTH receptors and their gonadal gene expression pattern has been carried out. The present study describes the presence of two distinct GTH receptors in trout showing similarities with those of higher vertebrates but also differences in their structural determinants. *In vitro* functional studies demonstrate that rtLH specifically activates its cognate receptor (EC_{50}=117 ng/ml), whereas purified rainbow trout FSH (rtFSH) activates FSHR but also LHR at supraphysiological doses (EC_{50}=38 vs 598 ng/ml for FSHR and LHR respectively). The high doses of rtFSH required to activate LHR put into question the physiological relevance of this interaction. The use of heterologous chinook GTHs confirms the strong preference of each hormone for its cognate receptor. The gonadal expression pattern of the GTH receptor genes suggests that FSH may play an important role in regulating gonadal functions, not only at the early stages but also at the final stages of the male and female reproductive cycles, in addition to the LH pathway.

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Introduction

The control of the gonadal functions by two plasma heterodimeric glycoproteins (known as gonadotropins (GTHs) and secreted from the gonadotrophs in the anterior pituitary) is a general model in vertebrate reproduction. In fish, two distinct GTH-I and GTH-II have been purified, and the cDNA of the corresponding specific subunits cloned from several species including salmon (Trinh *et al.* 1986, Suzuki *et al.* 1988, Sekine *et al.* 1989, Swanson *et al.* 1989). Based on their molecular structures and physiological effects, a new nomenclature has emerged and fish GTH-I and GTH-II are now referred to as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) respectively.

In fish, GTHs are differentially secreted in the plasma during the reproductive cycle suggesting that they have specific functions (Prat *et al.* 1996, Breton *et al.* 1998, Gomez *et al.* 1999, Sohn *et al.* 1999). FSH may play a determinant role in regulating early gametogenesis. In females, the specific role of FSH has been described with respect to the recruitment of oocytes into the secondary (vitellogenic) growth phase as well as in vitellogenin uptake (Tyler *et al.* 1991, 1997). In males, FSH induced an active spermatogonial proliferation in testicular explants cultured *in vitro* (Remacle 1976, Loir 1999) and has recently been shown to be involved in Sertoli cell proliferation (Schulz *et al.* 2005). In contrast, LH is the major regulating factor of late gametogenesis during oocyte maturation and ovulation (Jalabert 1976). In salmonids, LH alone stimulates production of the maturation-inducing steroid in the granulosa cells (17,20β-dihydroxy-4-pregnen-3-one), thus inducing germinal vesicle breakdown (Planas *et al.* 2000).

In addition, *in vitro* studies highlighted the ability of both hormones to stimulate steroid production in both ovaries and testis, although their potency differs and is mainly related to gonadal maturation (Swanson *et al.* 1991, Planas & Swanson 1995).

As in other vertebrates, once released in the plasma, GTHs bind to membrane-bound receptors expressed on the somatic cells of the gonads. The binding of LH to high-affinity receptors was first demonstrated in the salmonid ovary (Salmon *et al.* 1984, Breton *et al.* 1986, Kanamori *et al.* 1987) and the trout testis (Le Gac *et al.* 1988). Evidence for two distinct binding activities in the fish ovary originated from other binding studies (Yan *et al.* 1992, Miwa *et al.* 1994). In the salmon ovary, ligand-binding assays on gonadal tissue sections followed by autoradiography localized a type I receptor (presumably FSHR) on both thecal

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and granulosa cells, whereas a type II receptor (presumably LHR) was identified on granulosa cells only. In the testis, type I and type II receptors were localized on cells lining the tubules and on interstitial cells respectively (Miwa *et al.* 1994). Subsequently, the presence of two distinct GTH receptors was confirmed by the molecular cloning of two different cDNA in several fish species: salmon (Oba *et al.* 1999*a*,*b*, Maugars & Schmitz 2006), catfish (Bogerd *et al.* 2001, Kumar *et al.* 2001*a*,*b*, Vischer & Bogerd 2003), and zebrafish (Kwok *et al.* 2005, So *et al.* 2005). These membrane receptors belong to the G-protein-coupled receptor (GPCR) superfamily and, in particular, to the subfamily of glycoprotein hormone receptors, characterized by a larger extracellular N-terminal domain, a seven helical transmembrane region, and a short intracellular tail.

Although the overall structure is similar to that described in all other vertebrate species, including reptiles, birds, and mammals, marked differences exist with regard to the hormonal specificity of these receptors toward the fish GTHs. Binding studies with highly purified fish GTHs showed cross-reactivity. In coho salmon, type I receptor (FSHR) was able to bind preferentially to FSH and to a lesser extent to LH (Miwa *et al.* 1994). In carp, purified GTH receptors bound preferentially to their cognate GTH but a moderate overlapping recognition was described (Basu & Bhattacharya 2002). Depending on the species, a promiscuous activation of one or the other fish GTH receptors was also reported in functional studies using mammalian cell lines expressing fish receptors. In African catfish, recombinant cfFSH and cfLH activated FSHR with a similar biopotency (Bogerd *et al.* 2001, Vischer & Bogerd 2003, Vischer *et al.* 2003), whereas in amago salmon, only FSH was able to activate FSHR (Oba *et al.* 1999*a*). However, hormonal specificity may also depend on the origin of the GTHs. In zebrafish, bovine FSH activated FSHR and LHR, whereas bovine LH specifically activated LHR (Kwok *et al.* 2005). In contrast, recombinant zebrafish FSH stimulated only FSHR, whereas recombinant LH stimulated both FSHR and LHR (So *et al.* 2005).

In summary, data from the binding and functional studies do not allow one to draw a general conclusion on the responsiveness of the piscine receptors to GTHs, even in salmonids, and further studies from other fish species or strains are worthwhile.

Besides the functional characterization, our knowledge of the expression pattern of the GTH receptor genes is scarce and limited to a few fish species including catfish (Kumar *et al.* 2001*a*,*b*), zebrafish (Kwok *et al.* 2005), tilapia (Oba *et al.* 2001), and salmonids (Campbell *et al.* 2006, Kusakabe *et al.* 2006).

In the present study, we report the isolation of two distinct cDNA encoding rainbow trout FSH and LH receptors. Analysis of the amino acid sequences of the fish GTH receptors shows a similar, but not identical, structure to that found in other vertebrates. The expression patterns of the corresponding transcripts have been determined during the male and female reproductive cycles. Distinct temporal expression patterns have been observed in female and male trout. The functional specificity of the trout GTH receptors was studied in transient transfected heterologous cells using purified rainbow trout GTHs (rtFSH and rtLH). Although an rtFSH-induced responsiveness of LHR was observed at supraphysiological doses, we demonstrated that rtLH and rtFSH activate their cognate receptor preferentially.

Materials and Methods

Animal and tissue collection

Male and female rainbow trout (*Oncorhynchus mykiss*) from a fall spawning strain were bred during their first reproductive season at the INRA/PEIMA fish farm (Sizun, France) and held under natural photoperiod and temperature. Monthly sampling was carried out to collect gonads at different gonadal maturation stages. Female gonadal maturation stages were determined as described previously (Jalabert 1976). Trout were anesthetized in 2-phenoxyethanol (0·03% v/v) and ovulation was checked every 3 days. A sample of ovulated females was manually stripped, and kept for 5–15 days after detection of ovulation. Fish were killed by cervical transection and gonads were dissected out of the body cavity under sterile conditions. A piece of the gonads was fixed in Bouin's solution and the rest frozen in liquid nitrogen and stored at −70 °C, until RNA extraction. For the two full-grown oocyte stages (end of vitellogenesis and maturation), the ovary was deyolked as described previously (Garczynski & Goetz 1997). The stage of male gonadal maturation was determined by histological analysis after Regaud's hematoxylin/orange G staining (Gabe 1968). Male gonadal stages were determined as described previously (Billard & Escafffre 1975). Gonadal stage I corresponds to immature male, stage II to active spermatogonial proliferation, stage III to meiosis onset, stage VI to full spermiogenesis, stage VIII to spermiation, and finally stage IX to post-spermiation.

Cloning of GTH receptor cDNA

To isolate full-length cDNA encoding GTH receptors in trout, known amino acid sequences, corresponding to glycoprotein hormone receptors, were aligned. Two conserved motifs spanning the third extracellular loop (KVSICLP) and the seventh transmembrane α-helix (PFLYAI) of these GPCR were chosen to design the forward el2 and reverse el3 degenerated primers. RT-PCR was carried out from testis cDNA and a 352 bp DNA fragment, called the FSHR probe in the present study, was isolated and sequenced. This probe was used to screen, as described previously (Sambroni *et al.* 2001), a stage III–IV testicular cDNA library, constructed in λZAP II vector (Stratagene, La Jolla, CA, USA). A single positive clone named FSHR B8 (1337 bp) was obtained with the FSHR probe but was lacking the 5′ extremity of the open reading frame. To complete the 5′ end of the open reading frame, a 5′ RACE-PCR was performed using the rapid amplification of cDNA ends, version 2.0 and the high fidelity elongase (GIBCO BRL Life Technologies).

Briefly, 1 μg total RNA was reverse transcribed with a specific primer Asn172 (Table 1) in order to synthesize the first strand cDNA. After removal of RNA template and purification of the first strand cDNA, an oligo-dC tail was added. The tailed cDNA was amplified with the AAP and Asn136 primers. This allowed us to add 1050 bp upstream from the 5′ end of the FSHR B8 clone. Completion of the open reading frame was carried out by RT-PCR with the RI-1 forward primer design from the 5′ untranslated region of the amago salmon sGTH-RI cDNA (AB030012) and Asn2-specific reverse primer.

For LHR cDNA molecular cloning, a partial cDNA fragment was first obtained by RT-PCR from reverse-transcribed testicular RNA (stage II) with RII-2 and Asn519 primers (Table 1) designed according to the amago salmon (*Oncorhynchus rhodurus*) sGTH-R cDNA (AB030005). The PCR product (440 bp) was cloned into the pCR2.1TOPO cloning vector (Invitrogen) and is called the LHR probe in the present study. A full-length cDNA (clone 2b-1) was isolated by screening the rainbow trout testicular cDNA library with the LHR probe as described above.

The nucleotide sequences of the cDNA encoding either the FSHR (2783 bp) or the LHR (2756 bp) have been submitted to GenBank under the accession numbers AF439405 and AF439404 respectively.

RNA extraction and reverse transcription

Gonadal tissue was homogenized in Trizol reagent (Invitrogen) at a ratio of 1 ml/100 mg tissue. Total RNA was extracted according to the manufacturer's instructions and resuspended in nuclease-free water. Two micrograms of total RNA were reverse transcribed in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV; Promega) according to the manufacturer's instructions. Reverse transcription of the total RNA was carried out in the presence of either oligo-dT_{18} (0·5 μg/μg total RNA) for the molecular cloning of the GTH receptor cDNA or in the presence of random hexamers (0·5 μg/μg total RNA) for the quantitative real-time PCR. The reverse transcription reaction was performed in a total volume of 25 μl, at 37 °C for 1 h and 15 min, followed by a 15-min incubation at 70 °C. Control reactions were run without MMLV reverse transcriptase and used as negative controls in the quantitative real-time PCR study.

Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using an i-Cycler iQ (Bio-Rad). The primers used in the PCRs were 28SFw1/28SRv1, RFSHICF2/RFSHICR2, and RLHICF2/RLHICR2 to amplify the 28S rRNA (reference gene), FSHR, and LHR transcripts respectively (Table 1). At least one of the specific PCR primers was designed on an exon/intron boundary of the FSHR and LHR genes. Reverse transcription products were diluted to 1:40 (or 1:2000 for 28S rRNA) and 5 μl were used for each real-time PCR. PCRs were carried out using the qPCR Mastermix Plus for SYBR Green I kit (Eurogentec, Angers, France), according to the manufacturer's instructions. Briefly, a 20 μl reaction mix was set up with 5 μl reverse-transcribed RNA sample, 5 μl appropriate primers (2·4 μM each), and 10 μl 2× reaction buffer. Thermal cycling was conducted at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was analyzed in duplicate. A pool of testicular reverse-transcribed RNA originating from stage II testis was serially half diluted and used as standard curve to check the linearity of the amplification and to calculate primer set efficiency. Primers

Table 1 Primers used in PCR experiments

	Sequences
Primers	
el2	5′-AAGGTSAGCATTTGCCTSCC-3′
el3	5′-AATMGCCTATAGCAAAGG-3′
Asn172	5′-GAAGCTCGTGAAGATGAGGATGGC-3′
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′
Asn136	5′-CATGCGCGTTTCAGCACTGGC-3′
RI-1	5′-GGTGCTGGGACGAGAAAAAGACA-3′
Asn2	5′-CGGCCCCTCTGCTCCTTTGAA-3′
RII-2	5′-GAAAAGTTGGATAATTCAATG-3′
Asn519	5′-ATCCCACTGTTGGATATGSWCARRTA-3′
RFSHEXPFw2	5′-AAGCTTGAGATGATGAAGATGAAGAAGAAT-3′
RFSHOZEXPRv	5′-AACTGGTTCAAATCAACCAAGATCTAGA-3′
RLHEXPFw	5′-GGATAATTCAATGATGTCGATA-3′
RLHEXPRv2	5′-TTTCAGGTATAGGGTGCCATT-3′
RFSHICF2	5′-TCAGTCACCTGACGATCTGCAA-3′
RFSHICR2	5′-TCCTGCAGGTCCAGCAGAAACG-3′
RLHICF2	5′-CTTCTCAACCTCAATGAAATCTTC-3′
RLHICR2	5′-GGATATACTCAGATAACGCAGCTT-3′
28SFw1	5′-TGTGAACAGCAGTTGAACATGG-3′
28SRv1	5′-ATCTGAACCCGACTCCCTTT-3′

set efficiencies were 96·5±19·2% for FSHR, 93·4±13·2% for LHR, and 84·2±10·5% for 28S rRNA. Correlation coefficients of the standard curves were 0·996±0·001, 0·997±0·001, and 0·996±0·02 for FSHR, LHR, and 28S rRNA respectively. No amplification was observed from non-reverse-transcribed sample RNA (from testis at stages I–III, and VIII), indicating the absence of contaminant such as genomic DNA. Specificity of the PCR product was determined from the melting curve analysis (10 s holding followed by a 0·5 °C increase, repeated 80 times, and starting at 55 °C). The mean cycle threshold (C_T) was calculated for each sample using the iCycler software (Bio-Rad). The relative abundance of target cDNAs was first normalized with 28S rRNA using the well-established delta C_T method (Livak & Schmittgen 2001). No significant ($P>0·05$) difference in the reference 28S rRNA gene expression was observed between stages of gonadal maturation either in males or in females. Data were analyzed using the nonparametric Mann–Whitney *U* test of the Statistica software (Statsoft, France).

Functional characterization of rainbow trout FSHR (rtFSHR) and LHR

The putative open reading frame, including the stop codon of each cDNA, was PCR amplified using RFSHEXPFw2/RFSHOZEXPRv and RLHEXPFw/RLHEXPRv2 primer sets. The resulting PCR products were cloned into the pcDNA 3.1/V5-His-TOPO expression vector (Invitrogen) upstream from the polyadenylation site of the bovine growth hormone gene and downstream the cytomegalovirus (CMV) promoter. The inserts were entirely checked by DNA sequencing on both strands. To test the functionality of FSHR and LHR, we expressed each receptor in COS-7 cells that were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) newborn calf serum. In total, 500 000 cells, seeded onto six-well plates, were cotransfected with either pcDNA 3.1/V5-His-FSHR or pcDNA 3.1/V5-His-LHR (500 ng/well) together with 1 μg/well of the cAMP-responsive reporter construct pCRE-Luc (Stratagene) and 50 ng/well pCMV β-galactosidase (Clontech) using 3 μl/well of FuGENE6 reagent (Roche Applied Science). Note that DNA quantity was adjusted to 2 μg using the pGEM-T vector (Promega). Seven hours after transfection, cells were trypsinized and replated on 24-well plates overnight. Twenty-four hours after transfection, cells were stimulated with purified GTHs for six hours. Incubations were stopped by washing the cells twice with PBS 1× and adding 100 μl 1× cell lysis buffer (Promega Corporation). Cells were incubated for 15 min under agitation (100 ***g***) at room temperature, and frozen at −20 °C until luciferase activity was measured from 20 μl lysates using the luciferase assay kit (Promega). The β-galactosidase activity was determined according to the manufacturer's instructions (Promega) from 30 μl cell lysates. Each stimulation was performed in triplicate and each experiment repeated at least twice. The hormone concentrations inducing half-maximal stimulation (EC_{50}) were calculated using the GraphPad Prism 4 software package (GraphPad Software Inc., San Diego, CA, USA). Rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) GTHs were purified using metal ion affinity chromatography and dye–ligand chromatography (Govoroun *et al.* 1997). The homogeneity and specificity of the GTH preparations were checked by reverse-phase high pressure liquid chromatography (Govoroun *et al.* 1997) and RIA (Govoroun *et al.* 1998).

Sequence analysis

The amino acid alignments of the sequences with other known G-protein-coupled receptors were performed using CLUSTALW and BioEdit shareware. The signal peptide cleavage site was predicted at the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The seven membrane-spanning regions were predicted at the server TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0). Prediction of the Ser, Thr, and Tyr phosphorylation sites was carried out at http://www.cbs.dtu.dk/services/NetPhos/. The potential *N*-glycosylation sites were predicted at http://www.cbs.dtu.dk/services/NetNGlyc/.

Statistical analysis

Data were analyzed using the nonparametric Mann–Whitney *U* test of the Statistica software based on the ranking method.

Results

Cloning of transcripts encoding proteins related to the GTH receptors

To characterize the rainbow trout GTH receptors, the molecular cloning of the corresponding transcripts was undertaken. Two full-length cDNA were isolated from trout testis as described in the Materials and Methods section. Figure 1 shows the deduced amino acid sequence from the open reading frame of each cDNA. The encoded proteins share poor overall homology (42% identity/56% homology). A search for conserved functional domains and motifs showed that the putative proteins have a structure similar to that of the GPCR superfamily and, in particular, to the glycoprotein hormone receptors that belong to the δ-subfamily (Fredriksson *et al.* 2003). Amino acid sequence alignment and phylogenetic analyses with known GPCR showed strong overall homology with the vertebrate GTH receptors (Fig. 2). The distinct cDNA segregated into two different clades, corresponding to each of the GTH receptor types. Therefore, the cDNA were named according to the homologous GTH receptor type. The FSHR cDNA length is 2783 bp and the open reading frame encodes 658 aa corresponding to a 73·7 kDa translated protein. The LHR cDNA is 2756 bp and harbors a 728 aa open reading frame corresponding to an 80·75 kDa translated protein.

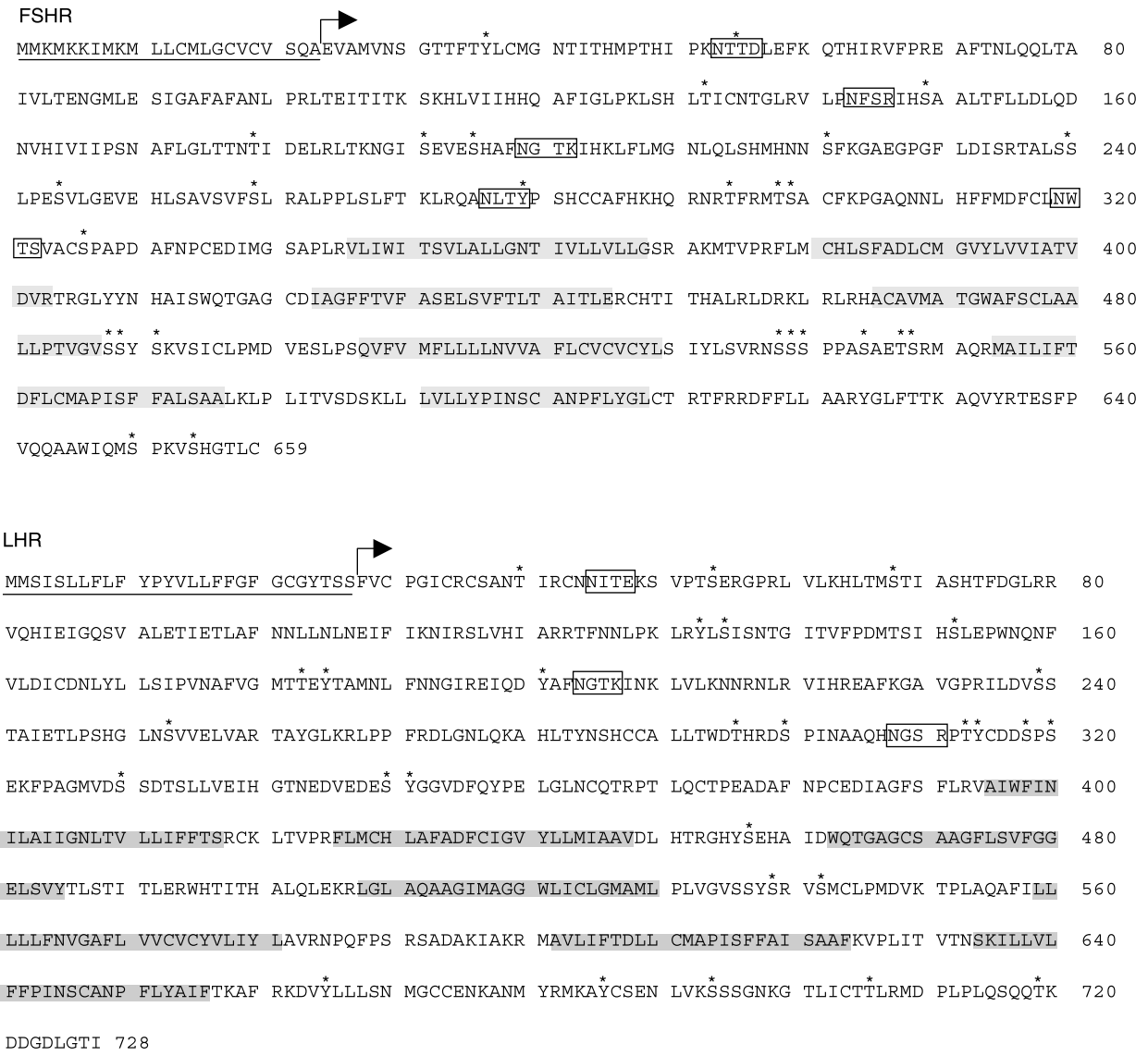
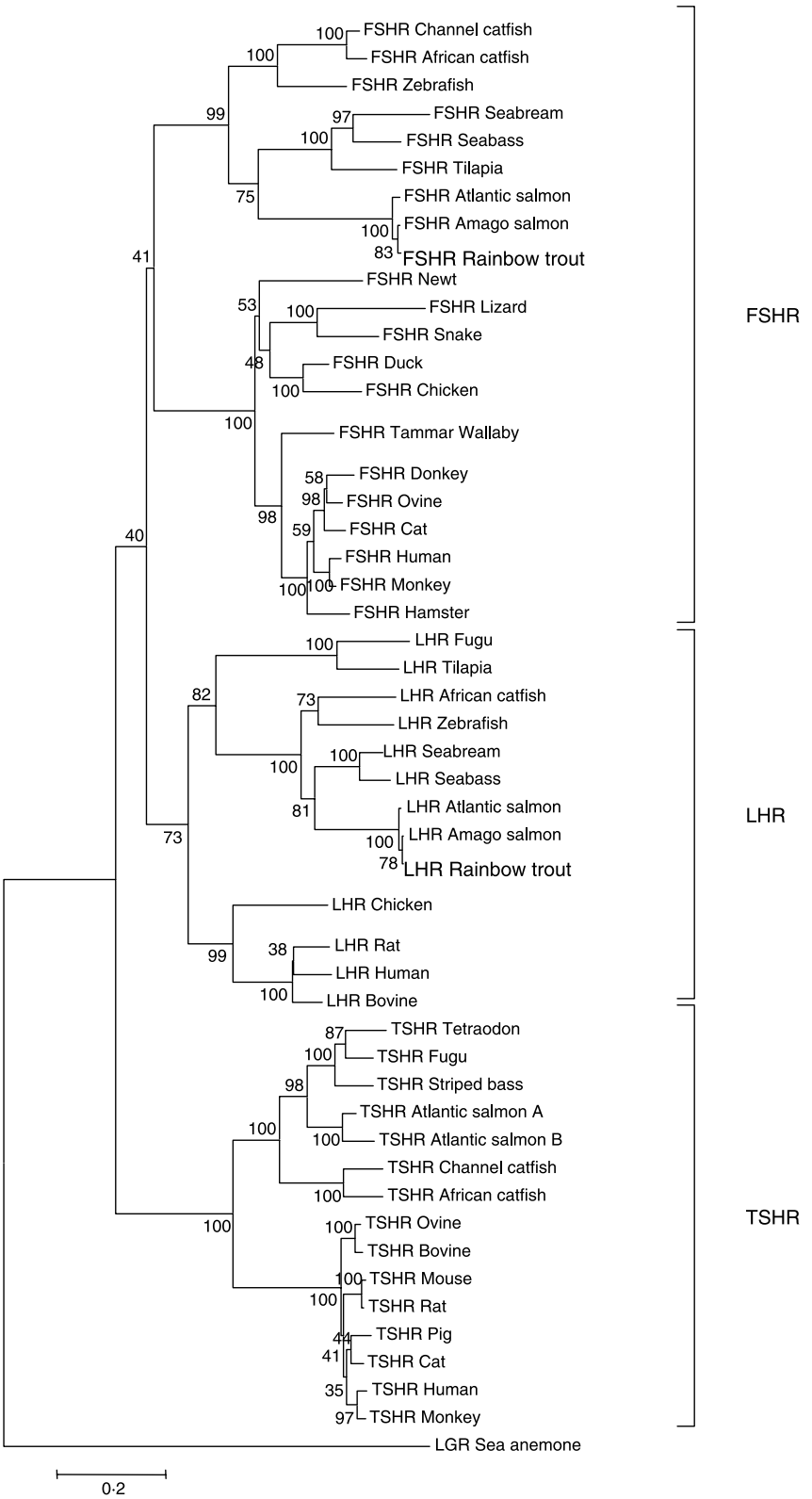


Figure 1 Amino acid sequences of rainbow trout FSHR (GenBank accession number AAQ04551) and LHR (GenBank accession number AAQ04550). The predicted signal peptides at the N-terminal are underlined and the putative cleavage sites are indicated with a broken arrow. Boxes and asterisks represent the potential *N*-glycosylation and phosphorylation sites respectively. The seven transmembrane helices are shown in grey.

The highest similarity for both trout GTH receptors (99%) is displayed by the Atlantic salmon (*Salmo salar*) and the amago salmon (*O. rhodurus*) GTH receptors. Homologies with other FSHR among teleost fish remain rather high ranging from 75 to 80%. The overall homology of trout FSHR with other vertebrate counterparts decreases to around 70% (human, reptiles, batrachians, birds). Interestingly, LHR appears to be better conserved among fish species (from 83 to 87%) with the exception of the tilapia, *Oreochromis niloticus* (71%), and the channel catfish, *Ictalurus punctatus* (69%). A similar homology with other vertebrates (mammals, birds) is observed which ranges from 69 to 73%.

A structural analysis of the putative rtFSHR and rtLHR amino acid sequences indicates the presence of three main functional regions: extracellular, transmembrane, and intracellular domains. A large extracellular domain that displays similar features to the leucine-rich glycoprotein receptors (LGR) is located at the N-terminal end. This extracellular domain includes a 23 (rtFSHR) to 27 (rtLHR) amino acid region that is highly hydrophobic and encompasses a putative signal peptide, as revealed using the sliding window/matrix scoring method and -1, -3 rule for signal peptide prediction. A cluster of nine repeated sequences is observed on each receptor (Fig. 3). These sequences are related to imperfect


FSHR Channel catfish
FSHR African catfish
FSHR Zebrafish
FSHR Seabream
FSHR Seabass
FSHR Tilapia
FSHR Atlantic salmon
FSHR Amago salmon
FSHR Rainbow trout
FSHR Newt
FSHR Lizard
FSHR Snake
FSHR Duck
FSHR Chicken
FSHR Tammar Wallaby
FSHR Donkey
FSHR Ovine
FSHR Cat
FSHR Human
FSHR Monkey
FSHR Hamster
LHR Fugu
LHR Tilapia
LHR African catfish
LHR Zebrafish
LHR Seabream
LHR Seabass
LHR Atlantic salmon
LHR Amago salmon
LHR Rainbow trout
LHR Chicken
LHR Rat
LHR Human
LHR Bovine
TSHR Tetraodon
TSHR Fugu
TSHR Striped bass
TSHR Atlantic salmon A
TSHR Atlantic salmon B
TSHR Channel catfish
TSHR African catfish
TSHR Ovine
TSHR Bovine
TSHR Mouse
TSHR Rat
TSHR Pig
TSHR Cat
TSHR Human
TSHR Monkey
LGR Sea anemone
0·2
FSHR
LHR
TSHR

leucine-rich repeats (LRRs) and most likely form a succession of β-strands and α-helices, organized into a horseshoe-shaped structure. These nine LRRs, conserved in sequence and position between rtFSHR and rtLHR, are similar in length and sequence to the typical LRR (Kajava 1998). Interestingly, the proximal cysteine-rich flanking region that normally links the N-terminal end of the protein to the LRR clusters in mammalian FSHR and LHR, is not found in the rtFSHR sequence. This structural change is observed in salmoniform and perciform FSHR, whereas in zebrafish and catfish, only two of the four cysteine residues liable to form a disulfide bond are present (Fig. 4). The distal cysteine-rich domain that precedes the transmembrane domain is present in both receptors, and is close to the C-terminal cysteine-containing flanking domain 3 (CF3) domain consensus described by Kajava (1998).

The extracellular domain displays five and three potential *N*-glycosylation sites in rtFSHR and rtLHR respectively (Fig. 1). Only the glycosylation motif (NGT) is conserved in the three glycoprotein hormone receptors out of all species studied so far.

As observed in other vertebrate GTH receptors, the transmembrane domain is the most conserved in terms of sequence and size and is composed of seven hydrophobic regions, forming a tertiary structure of short α-helices. Spacer regions that have been involved in extracellular and intracellular loops are also well conserved. Finally, the C-terminal end forms an intracellular domain. The intracellular domain of the rtLHR includes five putative tyrosine or serine/threonine kinase phosphorylation sites. The intracellular domain of the rtFSHR is shorter than that of the rtLHR (52 aa versus 72 aa) and this feature is also observed in other fish species. In addition to the shorter size, only two putative conserved phosphorylation sites for serine/threonine kinases are found.

Pharmacological characterization of rainbow trout GTH receptors

The functionality of the trout putative GTH receptors was studied by transient transfection assays in COS-7 cells expressing either one of the receptors. Since it is well established that GTHs induce intracellular cAMP production upon binding to their receptors, an indirect quantification of intracellular cAMP production was carried out using a cAMP-responsive luciferase reporter gene. The high concentration (800 ng/ml) of purified fish GTHs used in this study did not modulate reporter gene expression in the absence of GTH receptors (Fig. 5A). Transfection of the cells with increasing amounts of the expressing vectors (from 0 to 500 ng/well) showed that, in the absence of a ligand, both trout GTH receptors had no constitutive activity (Fig. 5B and C).

Transactivation data, obtained upon incubation in the presence of GTHs, are reported in Fig. 6 and maximal induction values are indicated in Table 2.

Regarding rtFSHR, a dose-dependent response curve was observed using rtFSH at a concentration of 25–1000 ng/ml (Fig. 6). A significant ($P<0{\cdot}05$) maximal 2·5-fold induction (Table 2) of the reporter gene expression was seen at 100 ng/ml. No reporter gene induction was observed in presence of rtLH. Similar results were obtained using chinook GTHs (cFSH and cLH). cFSH was able to activate rtFSHR to a similar level (2·4-fold induction) but at a higher dose compared with rtFSH (400 vs 100 ng/ml). cFSH potency appeared to be much lower with a calculated effective half-maximum concentration (EC_{50}) equal to 200 ng/ml versus 38 ng/ml for rtFSH. As observed for rtLH, cLH was not able to induce reporter gene expression regardless of the concentrations used (Fig. 6).

Regarding rtLHR, rtLH induced luciferase reporter gene expression in a dose-dependent manner from 25 to 1000 ng/ml (Fig. 6). The rtLHR was highly responsive, showing a significant ($P<0{\cdot}05$) 8·9-fold induction at 1000 ng/ml, and an EC_{50} of 117 ng/ml. Interestingly, high doses of rtFSH (1000–1600 ng/ml, data not shown) were also able to induce a significant ($P<0{\cdot}05$) and reproducible twofold induction of the reporter gene expression. However, the biopotency of rtFSH in activating rtLHR was 16-fold lower compared with that calculated for rtFSHR ($EC_{50}=598$ vs 38 ng/ml for rtLHR and rtFSHR respectively). The heterologous chinook GTHs were also tested. cLH induction resulted in a similar dose-dependent responsiveness to that obtained in the presence of rtLH. The biopotency of cLH was similar to that of rtLH with an

Figure 2 Phylogenetic analysis of rainbow trout gonadotropin receptors. Amino acid sequences of known or predicted glycoprotein hormone receptors were first aligned using CLUSTALW, and the resulting alignment was analyzed using the MEGA software. The analysis was carried out on full-length protein sequences using two different algorithms (minimum evolution and neighbour-joining methods). Similar trees were obtained but only the analysis performed by the neighbour-joining method is represented. The numbers beside the branches indicate bootstrap values from 100 replicates. The scale bar corresponds to estimated evolutionary distance units (MYA). The outgroup used to build the tree is the sea anemone leucine-rich repeat containing G-protein-coupled receptor (Z28332). TSHR sequence accession numbers are AY533543 (channel catfish), AY129556 (African catfish), AF239761 (striped bass), AB030954 (TSHRA, amago salmon), AB030955 (TSHRB, amago salmon), fugu (unpublished results), tetraodon (unpublished results), NM_012888 (rat), BC092523 (mouse), NM_001009410 (sheep), NM_174206 (bovine), AF218264 (cat), NM_214297 (pig), AY169400 (monkey), and AY429111 (human). LHR sequences are AF324540 (African catfish), AY424302 (zebrafish), fugu (unpublished results), AY642114 (sea bass), AF439404 (rainbow trout), AB030005 (amago salmon), AB041763 (tilapia), AJ579790 (Atlantic salmon), AY587261 (sea bream), NM_204936 (chicken), NM_012978 (Rat), U20504 (bovine), and NM_000233 (human). FSHR sequences are AY587262 (sea bream), AJ567667 (Atlantic salmon), AF285182 (channel catfish), AB030012 (amago salmon), AF439405 (rainbow trout), AY278107 (zebrafish), AY642113 (sea bass), AB041762 (tilapia), AJ012647 (African catfish), AY189696 (snake), AB005587 (newt), AJ292553 (lizard), NM_205079 (chicken), AY099289 (duck), AY082002 (tammar wallaby), AY509907 (hamster), AY521181 (cat), U73659 (donkey), NM_001009289 (sheep), NM_214386 (pig), XM_001114171 (monkey), and M65085 (human). Note that rainbow trout cDNA encoding glycoprotein hormone receptors segregate into two distinct clades, corresponding to the FSHR and LHR genes.

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LHR        RGPRLVLKHLTMST-IASHTFDGLR                 56-  79
FSHR       T-TDLEFKQTHIRV-FPREAFTNLQ                 54 -  76

LHR        RVQHIEIGQSVALETIETLAFNNLL                 80 - 104
FSHR       QLTAIVLTENGMLESIGAFAFANLP                 77 - 101

LHR        NLNEIFIKNIRSLVHIARRTFNNLP                105 - 129
FSHR       RLTEITITKSKHLVIIHHQAFIGLP                102 - 126

LHR        KLRYLSIS-NTGITVFPDMTSIHSLEPWN            130 - 157
FSHR       KLSHLTIC-NTGLRVLPNFSRIHSAA               127 - 151

LHR        QNFVLDICDNLYLLSIPVNAFVGMTT               158 - 183
FSHR       LTFLLDLQDNVHIVIIPS-AFLGLTTN              152 - 178

LHR        EYTAMNLFNNG-IREIQDYAFNGT                 184 - 206
FSHR       TIDELRLTKNG-ISEVESHAFNGT                 179 - 201

LHR        KINKLVLKNNRNLRVIHREAFKGAV                207 - 231
FSHR       KIHKLFLMGNLQLSHMHNNSFKGAE                202 - 226

LHR        GPRILDVSSTA-IETLP--SHGLN                 232 - 252
FSH        GPGFLDISRTA-LSSLPE-SVLGE                 227 - 248

LHR        SVVELVARTAYGLKRLPPFRDLG                  253 - 275
FSHR       -VEHLSAVSVFSLRALPPLSLFTK                 249 - 271

Cons LRR   xLxxLxLxxNx-LxxLPxxxFxx
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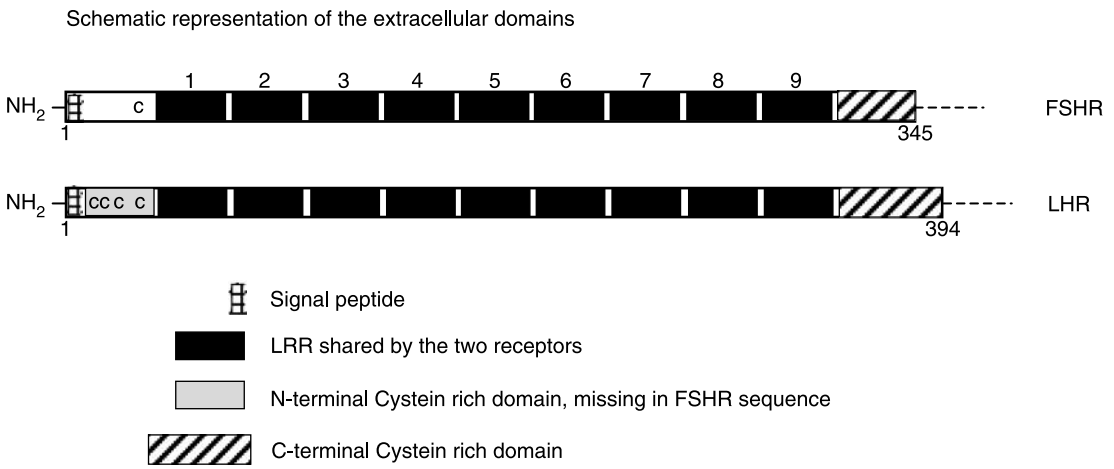

Figure 3 The nine repeat sequences found in the extracellular domains of rainbow trout FSHR and LHR are related to imperfect leucine-rich repeats (LRRs). Spaces have been added to improve the alignment. The consensus sequence of LRR (Kajava *et al.* 1995) is indicated below the alignments. A schematic of the extracellular domains is shown at the bottom which indicates the position of different structural motifs: signal peptide, the LHR N-terminal cysteine-rich flanking region containing four cysteine residues (c), the cluster of imperfect LRR numbered 1–9, and the C-terminal cysteine-rich flanking region. The amino acids delineating the ectodomain are indicated. Note the absence of the N-terminal cysteine-rich flanking region on rtFSHR.

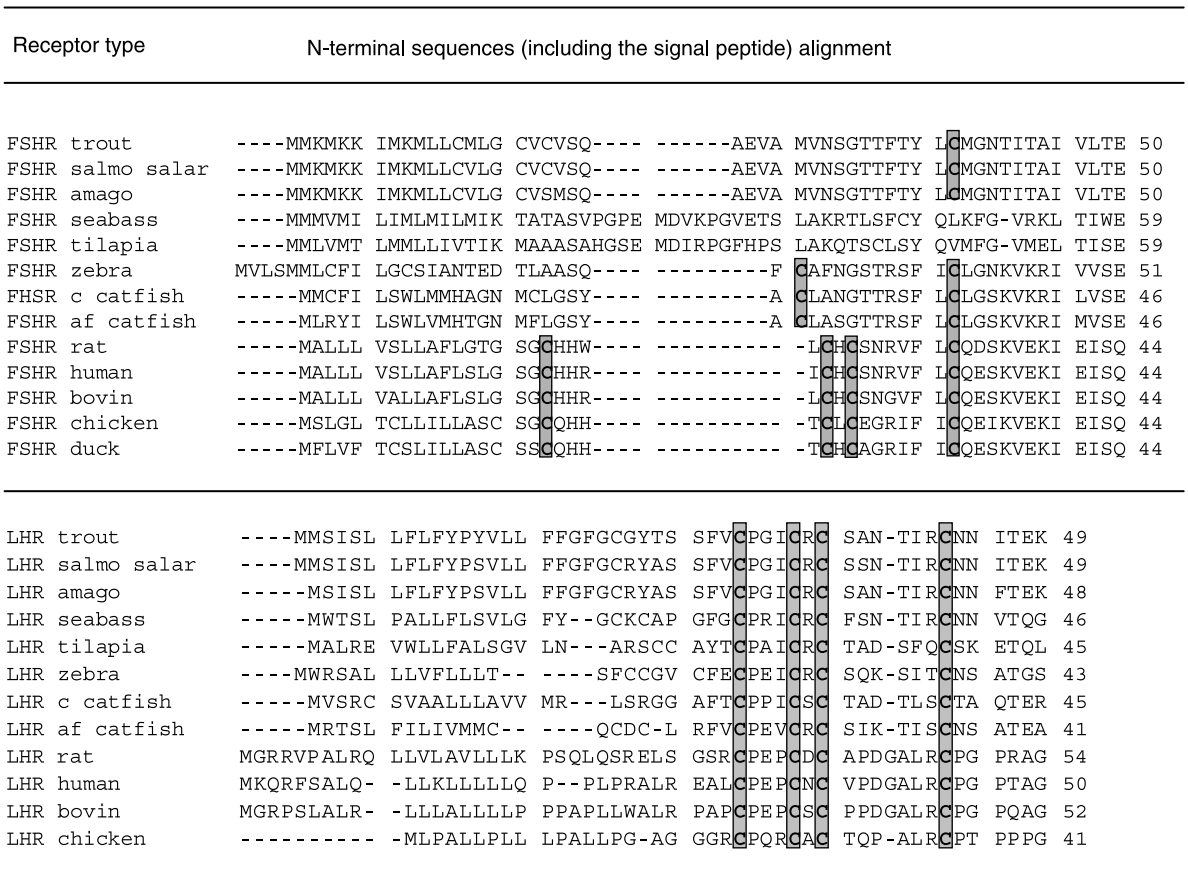

Figure 4 Alignment of the rtFSHR and rtLHR N-terminal cysteine-rich flanking regions with those of other piscine and mammalian species. Conserved cysteine residues are shown bold in grey boxes. c catfish, channel catfish; af catfish, African catfish.

EC_{50} equal to 98 vs 117 ng/ml for rtLH (Table 2), with an 11-fold maximal induction obtained at 400 ng/ml. Unexpectedly, contrary to rtFSH, cFSH had no significant effect on rtLHR transactivation at the tested doses.

In summary, functional data indicate that, in our assay system, rtLHR and rtFSHR showed a marked functional specificity to their cognate ligand.

Expression pattern of the trout GTH receptor genes in the gonads

To provide new insight into trout GTH receptor function, the expression pattern of their genes was studied over the annual reproductive cycle in female and male trout using quantitative real-time PCR. In females, rtFSHR and rtLHR transcripts were present in all stages studied (Fig. 7); however, different expression patterns were found for the two messengers prior to spawning (Fig. 7B). The relative abundance of rtLHR transcript significantly increased during the ovulation period (fivefold, $P<0{\cdot}05$). Unexpectedly, the relative abundance of rtFSHR peaked significantly ($P<0{\cdot}05$) earlier, just before spawning at final oocyte maturation and ovulation (sixfold compared with the end of vitellogenesis).

We previously showed an inverse evolution of GTH plasma levels after ovulation, dependent on whether the eggs were retained in the abdominal cavity (Breton *et al.* 1998). FSH secretion significantly increased after ovulation in fish without eggs, whereas in fish that retained eggs, FSH secretion remained constant. The LH profile was exactly the opposite, being significantly higher in fish with eggs compared with those that were stripped. In order to determine whether a modification in the plasma hormonal balance, induced after ovulation by egg removal, correlates with changes in GTH receptor gene expression, we examined the relative abundance of ovarian rtFSHR and rtLHR transcripts in ovulated females with or without eggs retained in the abdominal cavity after ovulation (Fig. 7C). The relative abundance of rtFSHR transcripts significantly decreased after ovulation (fivefold decrease, $P<0{\cdot}05$). This decrease seemed to be slightly delayed in females that retained eggs in the abdominal cavity, but a significant difference between the two groups (twofold, $P<0{\cdot}05$) was only detected 10 days after ovulation. On the contrary, rtLHR transcript did not change significantly for up to 15 days after ovulation, and no significant difference was observed in females with or without eggs.

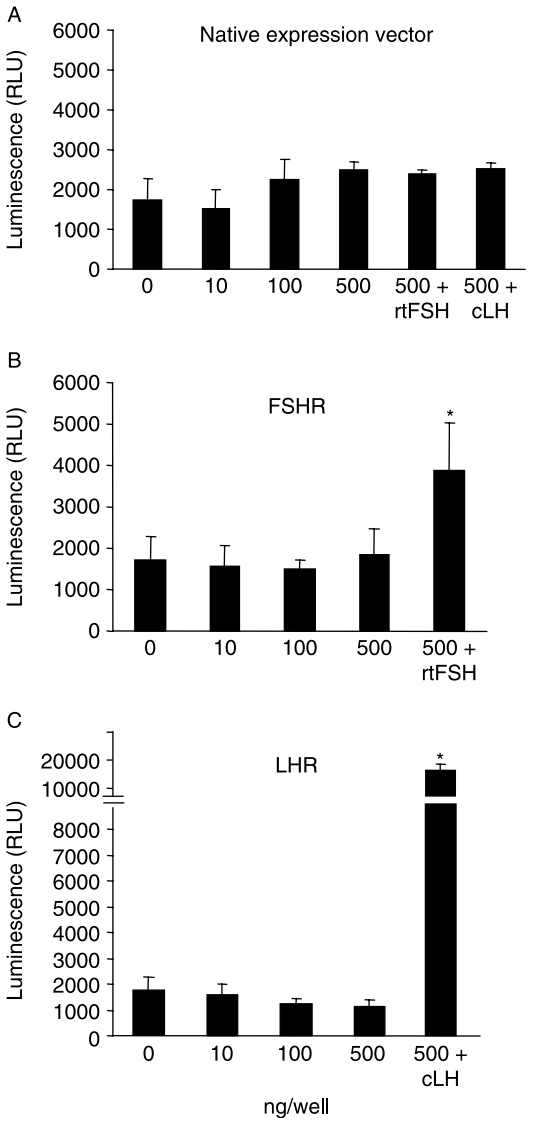

Figure 5 Analysis of the basal activity of rainbow trout gonadotropin receptors. COS-7 cells were cotransfected with increasing amounts (from 0 to 500 ng/well) of native (A) or trout gonadotropin receptor (B and C) expression vectors, together with the cAMP-responsive reporter construct pCRE-Luc. Cells were incubated in the absence of gonadotropins or were stimulated for 6 h in the presence of 800 ng/ml FSH (+rtFSH) or LH (+cLH) as a positive control. cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. Each data point represents the mean ± S.D. of triplicates of a representative experiment.

In males, rtFSHR and rtLHR transcripts were also present in all the studied stages and their expression followed a similar pattern (Fig. 8). In immature fish and during the gonial proliferation period (stages I and II), the relative abundance of both transcripts did not change significantly. Starting from meiosis onset to full spermiogenesis, there was a progressive and significant ($P<0{\cdot}05$) decrease in rtFSHR and rtLHR transcripts (8- and 12-fold decrease respectively). However, normalization of the data corrected for the reference gene, the gonadosomatic index and RNA extraction yields indicate no significant change in the rtFSHR and rtLHR messenger levels during spermatogenesis. Interestingly, independent of the normalization procedure used, both transcripts reached their maximum expression at spermiation (four- to fivefold increase for rtFSHR and rtLHR respectively compared with the immature gonadal stage I). In post-spawning males (stage IX), rtFSHR levels returned to nearly immature levels, whereas rtLHR messengers continued to be expressed at a level five times greater than that in immature fish. Normalizing the data for the gonadosomatic index and RNA extraction yields showed that the accumulation of the GTH receptor transcripts remained high in post-spawning/regressing male compared with immature fish.

Discussion

Structure of trout GTH receptors

In the present study, we identified two distinct cDNA encoding rainbow trout GTH receptors based on the analyses of their amino acid primary sequences and on *in vitro* functional studies with homologous and heterologous purified GTHs. The presence of high-affinity receptors for LH was previously demonstrated in male and female trout from *in vitro* binding assays to membrane preparations or purified GTH receptors (Quesnel & Breton 1993). Since no binding study has been carried out with purified trout FSH, clear evidence for a second distinct GTH receptor type in trout relied mainly on *in vitro* biological studies where purified FSH was shown to act on specific gonadal functions. Loir (1994, 1999) showed that purified rtFSH, but not rtLH, was a potent mediator of trout spermatogonia proliferative activity *in vitro*.

The presence of two genes encoding distinct GTH receptors in trout is in agreement with previous reports on other fish species, including amago salmon (Oba *et al.* 1999*a*,*b*), Atlantic salmon (Maugars & Schmitz 2006), African catfish (Bogerd *et al.* 2001, Vischer & Bogerd 2003), channel catfish (Kumar *et al.* 2001*a*,*b*), and zebrafish (Laan *et al.* 2002, Kwok *et al.* 2005). Analysis of the overall architecture indicates that the two trout receptors should be considered as new members of the GPCR superfamily and, in particular, to the glycoprotein hormone receptor (GpR) subfamily. Members of the GpR subfamily are characterized by a large N-terminal extracellular domain (ectodomain), a conserved seven α-helix transmembrane region, and a short C-terminal intracellular tail (Fredriksson *et al.* 2003). Each trout GTH receptor is highly similar to that of the few other fish counterparts identified so far, suggesting that two distinct GTH receptors are well conserved among euteleosts. Moreover, with the exception of the pCRR structural domain located at the N-terminal end of the mammalian FSHR, fish GTH receptors share similar structural motifs with their

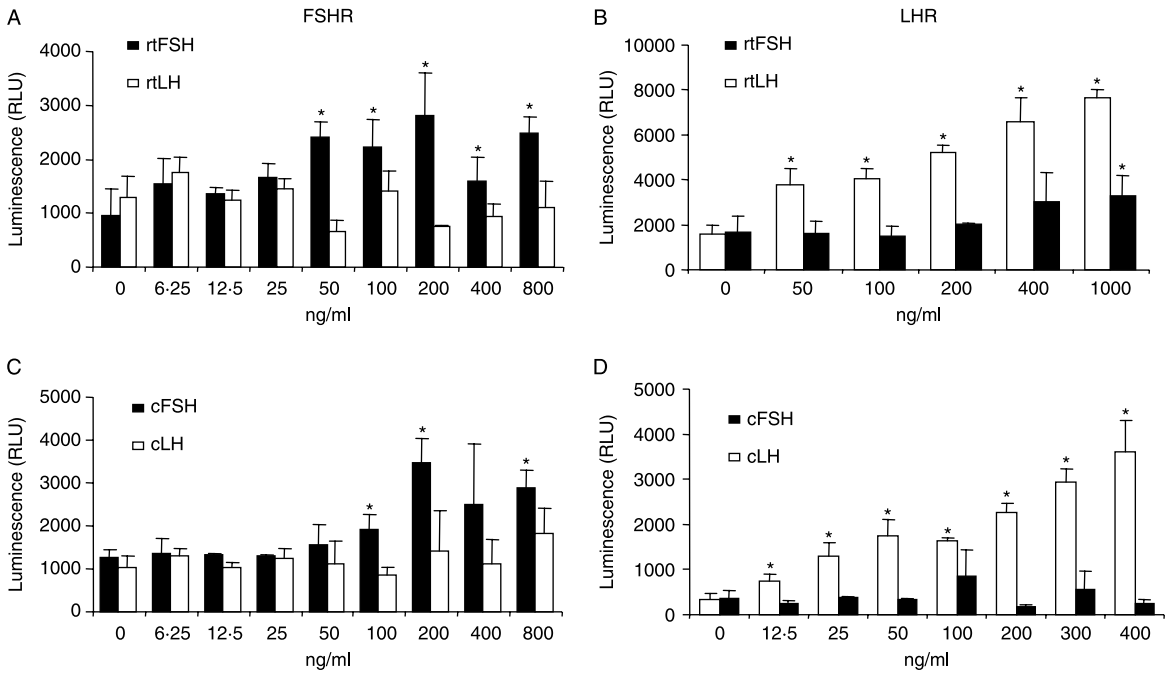

Figure 6 Transient expression and hormone responsiveness of rainbow trout FSHR and LHR. COS-7 cells were cotransfected with trout gonadotropin receptor expression vectors together with the cAMP-responsive reporter construct pCRE-Luc. Cells were incubated for 6 h with increasing doses of either rainbow trout (A and B) or chinook salmon gonadotropins (C and D). Hormone-induced cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. Each data point represents the mean ± S.D. of triplicates of a representative experiment.

reptilian, avian, and mammalian counterparts, indicating that they have been well conserved in all vertebrates during evolution. This assumption is also supported by the molecular phylogenetic analysis of the known vertebrate glycoprotein receptors, indicating that the two receptor types have emerged from a common ancestral gene.

In vitro *hormonal specificity of the trout GTH receptors*

To characterize their hormonal specificity at a functional level, trout GTH receptors were overexpressed in COS-7 cells together with a cAMP-responsive luciferase reporter gene and stimulated with an increasing amount of homologous or heterologous purified GTHs. As expected, the endogenous ligands were more potent in stimulating their cognate receptor, but the calculated EC_{50} required to transactivate rtFSHR (rtFSH: 38 ng/ml) and rtLHR (rtLH: 117 ng/ml) appeared to be higher than trout plasma GTH levels, which range from 2 ng/ml in juvenile to 15 ng/ml in sexually mature animals (Gomez *et al.* 1999). It is not known whether the apparent high EC_{50}, required to activate fish GTH receptors *in vitro*, is due to reduced biological hormone activities resulting from purification/conservation procedures.

Although rtFSH preferentially activates its own receptor, the rtFSHR maximal response to rtFSH (2·5-fold) was much lower than that of rtLH on rtLHR (about 9-fold). However, such a

Table 2 Effective half-maximum concentration (EC_{50}) and maximum induction calculated for each rainbow trout gonadotropin receptor. The maximum induction values are relative to the basal expression of the reporter gene measured in the absence of hormone. Data are the mean ± S.D. of three replicates of a representative experiment

	FSHR		LHR	
	EC_{50} ng/ml	Maximal induction	EC_{50} ng/ml	Maximal induction
Hormones				
rtFSH	38	2·5±0·1*	598	2·9±0·1*
cFSH	200	2·4±0·3*	na	1·0±0·2
rtLH	na	1·2±0·2	117	8·9±0·9*
cLH	na	1·8±0·6	98	11·0±2·1*

*Significant induction $P<0·05$; na, not applicable.

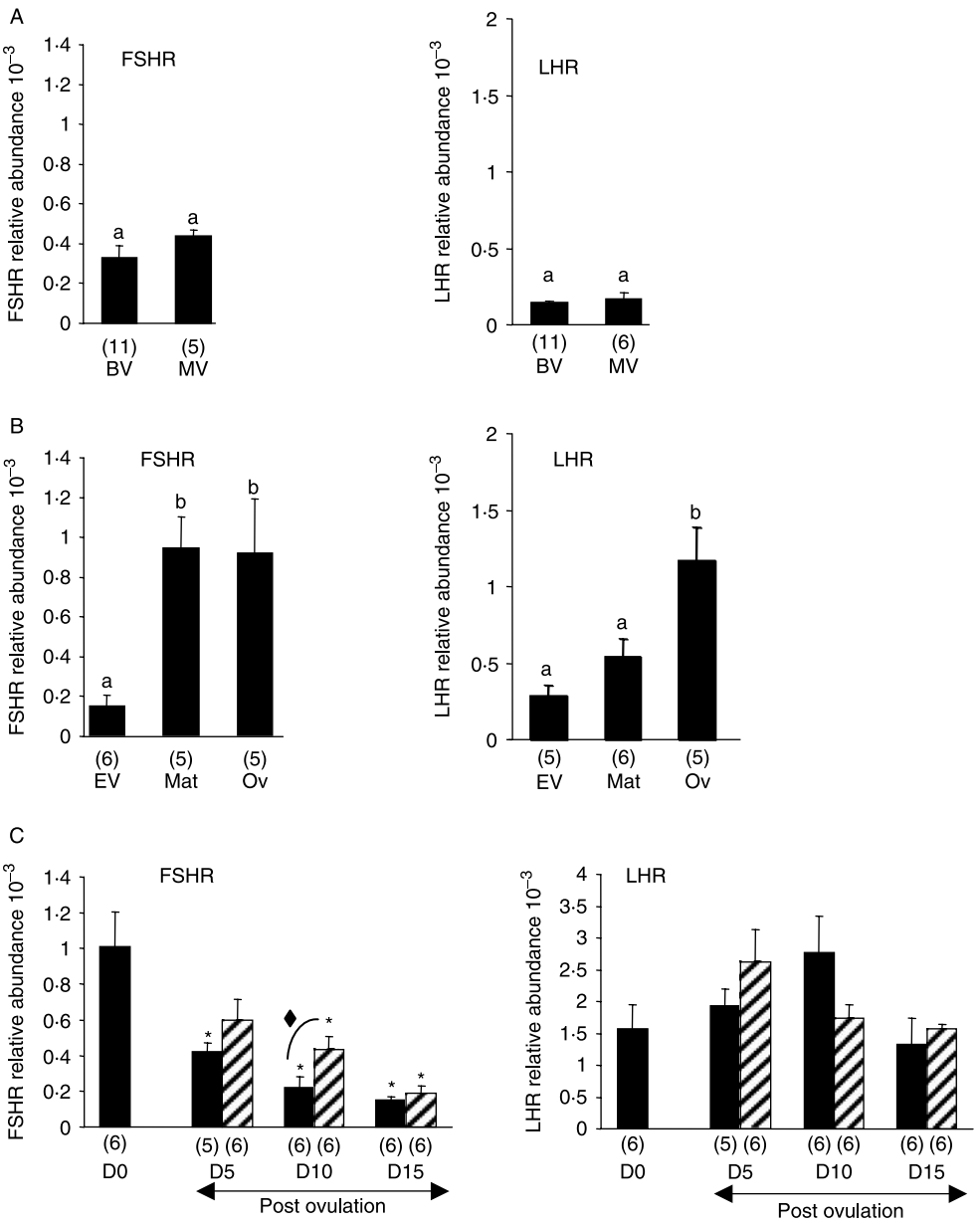

Figure 7 Expression profiles of female rainbow trout FSHR and LHR transcripts measured by quantitative real-time PCR during the first annual reproductive cycle from early to mid-vitellogenesis (A) and from the end of vitellogenesis to ovulation (B). Each gonadotropin receptor transcript was studied after the ovulation period (C). Expression levels were normalized to 28S rRNA. The black bars represent females stripped on the day of ovulation. Hatched bars represent females with eggs retained in the abdominal cavity for 5, 10, or 15 days after ovulation. * Asterisks represent significant difference ($P<0{\cdot}05$) compared with D0. The closed diamond symbol indicates significant differences ($P<0{\cdot}05$) between females with or without eggs retained in the abdominal cavity. Each data point represents the mean ± S.E.M. of different individual fish. Bars with different letters are significantly different ($P<0{\cdot}05$). Number of individual fish is indicated between brackets ($n=5$–11). BV, beginning vitellogenesis; MV, mid-vitellogenesis; EV, end vitellogenesis; Mat, maturation; Ov, ovulated females between 0 and 3 days.

weak FSH-induced response was previously reported in amago salmon using purified (Oba *et al.* 1999*b*) or recombinant (Ko *et al.* 2007) heterologous GTH regardless of the direct and indirect intracellular cAMP quantification systems used. Similar observations were also reported in catfish (Kumar *et al.* 2001*b*) and zebrafish (Kwok *et al.* 2005, So *et al.* 2005).

In our assays, rtLHR was activated not only by rtLH ($EC_{50}=117$ ng/ml) but also by rtFSH, albeit at doses at least

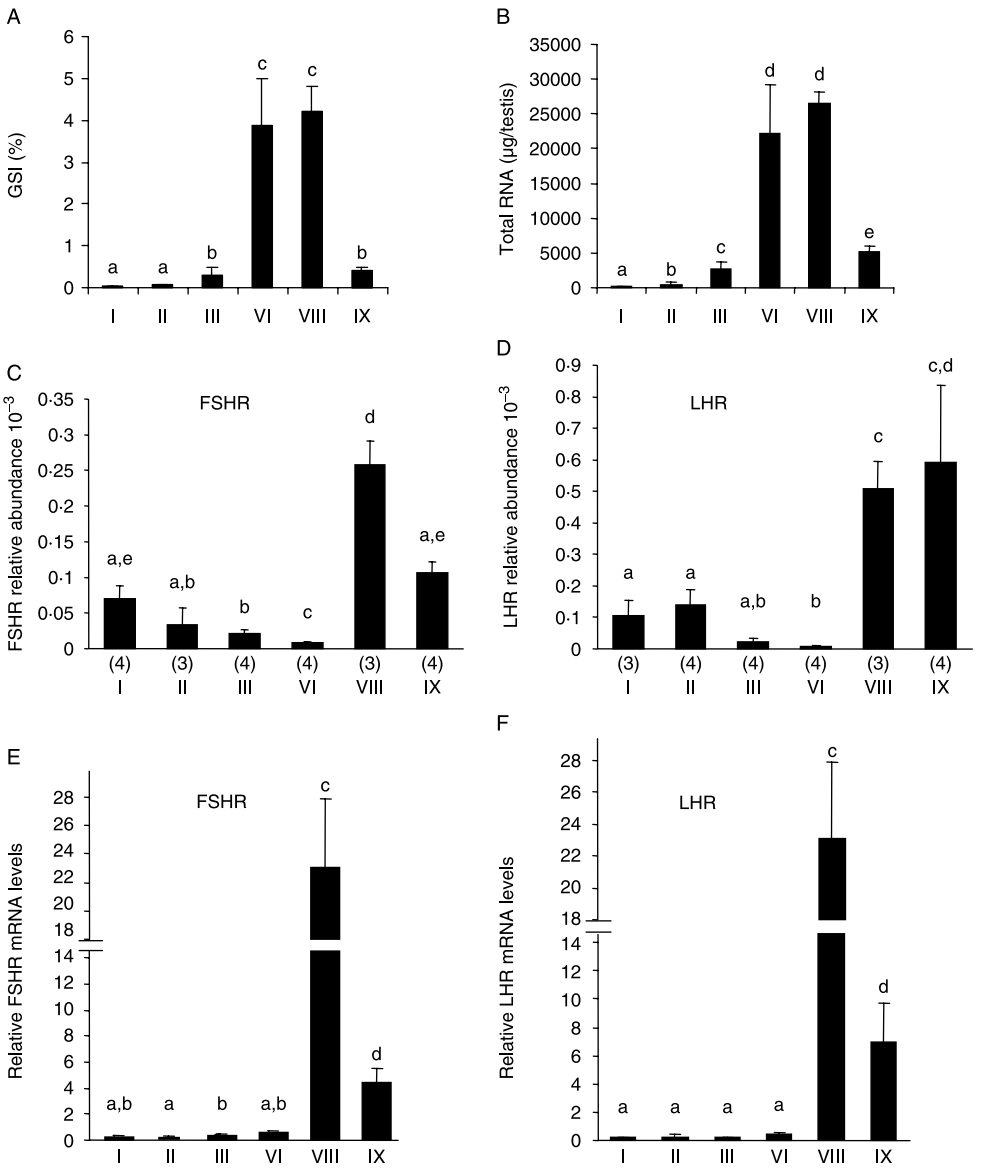

Figure 8 Expression profiles of male rainbow trout FSHR (C and E) and LHR (B and F) transcripts measured by quantitative real-time PCR during the first annual reproductive cycle: from immature (stage I) to post-spermiation (stage IX). The gonadosomatic index (A) and calculated total RNA content per testis (B) are presented. Expression data were normalized to 28S rRNA (C and D) or the gonadal RNA content (E and F) as described previously (Kusakabe *et al.* 2006). Each data point represents the mean ± S.E.M. (n=3–4). The number of individual fish is given in parentheses. Bars with different letters are significantly different (P<0·05).

five times higher (EC_{50} = 598 ng/ml). In addition, cells expressing rtLHR showed a high maximum response in the presence of rtLH (8·9-fold) that was significantly reduced in the presence of rtFSH (2·9-fold), independent of the dose used (up to 800 ng/ml). However, the activation of the rtLHR by homologous FSH was not observed upon stimulation by the heterologous chinook FSH. Contamination of the purified rtFSH fractions by rtLH may account for such an activation, although no trace of rtLH was detected by radio immunoassay in the purified rtFSH preparation (Govoroun *et al.* 1998). Other hypotheses include a weaker affinity of the heterologous hormone for the trout GTH receptors. This hypothesis is supported by the fact that the chinook FSH preparation used in the study specifically activated rtFSHR, but higher doses were required compared with the homologous rtFSH preparation. Unfortunately, we could not test very high concentrations because of limited availability of purified chinook GTHs.

Interestingly, the functional specificity of the trout GTH receptors is consistent with the reports in amago salmon, showing that, *in vitro*, a unique high concentration (5 μg/ml) of purified heterologous LH or FSH preferentially activates their cognate receptors (Oba *et al.* 1999*a*,*b*). These observations were recently confirmed using recombinant Manchurian trout GTHs (Ko *et al.* 2007). The prominent selective activation of the salmonid GTH receptors is rather unexpected since previous GTH-binding studies on membrane preparations from pacific salmon gonads demonstrated that FSHR bound both hormones, whereas LHR bound LH with a rather high selectivity (Yan *et al.* 1992). The difference observed between the results of the binding and activation studies suggests that LH binding to FSHR would result in a ligand/receptor complex unable to activate the adenylate cyclase in the COS cellular context. In addition, we cannot rule out the hypothesis that rtLH/FSHR complexes may activate other unknown signaling pathways.

In zebrafish, the functional specificity seems less prominent since recombinant zfFSH specifically stimulated its cognate receptor, whereas recombinant zfLH efficiently activated both GTH receptors (So *et al.* 2005). Similarly, in African catfish, recombinant cfFSH specifically stimulated cfFSHR whereas recombinant and purified cfLH were efficient in activating FSHR, although with a potency 10- to 20-fold lower (Vischer *et al.* 2003). Our study, together with other studies, indicates that the *in vitro* selective activation of the fish GTH receptors may differ depending on the fish species and/or order. In trout and amago salmon, FSHR and LHR would be specifically induced by their respective ligands, whereas in African catfish (Siluriforms) and zebrafish (Cypriniforms), which are two phylogenetically closely related species, FSHR would be efficiently activated by both hormones. In Siluriforms and Cypriniforms, but not in Salmoniforms, the N-terminal end of the FSHR exhibits two of four conserved cysteine residues present on a classical pCRR. These residues have been involved in disulfide bond formation and are part of a structural determinant required for efficient LH binding in mammals (Zhang *et al.* 1996). Whether these two conserved cysteine residues are involved in catfish and zebrafish FSHR folding and LH signal transduction remains to be investigated.

Expression of the GTH receptor genes in the gonad

In female trout, rtFSHR gene expression significantly increased at the maturation stage prior to ovulation. Such a FSHR gene expression pattern was also described in the tilapia (Oba *et al.* 2001) and zebrafish (Kwok *et al.* 2005). The physiological significance of high FSH levels and FSHR gene expression during oocyte maturation and spawning in salmonids remains puzzling. However, in mammals (Hoak & Schwartz 1980) and fish (Prat *et al.* 1996, Tyler *et al.* 1997), it has been proposed that FSH plays a role in follicle recruitment for the next reproductive cycle. Meanwhile, recombinant FSH injected into female rats induces not only follicle growth but also ovulation (van Cappellen *et al.* 1995). In trout, Bobe *et al.* (2003) found a positive correlation between the relative abundance of rtFSHR transcript and high follicular maturational competence. The rtLHR gene expression pattern in the ovary showed a progressive increase in LHR transcript abundance from the end of vitellogenesis to ovulation. Such a pattern is similar to that described in zebrafish (Kwok *et al.* 2005). The higher expression of GTH receptors in maturing and ovulated females is consistent with studies showing higher binding sites (Breton & Sambroni 1989) and high plasma GTH levels (Breton *et al.* 1998) in sexually mature females. Altogether, these results agree with the known functions of LH on ovarian steroidogenic activity, oocyte maturation, and spawning (Patino & Sullivan 2002).

In male trout, FSHR mRNA was detected in early gonadal maturation stages at Sertoli cell proliferation and differentiation (stages I and II). The presence of rtFSHR at the beginning of the cycle reinforces the idea that FSH could mediate Sertoli cell proliferation, as proposed previously in catfish and tilapia (Schulz *et al.* 2005). In the present study, the relative abundance of rtFSHR mRNA appeared to decrease progressively as the proportion of germ cells increased in the gonad (stages III and VI). A similar pattern was observed for the LHR transcript. Are these apparent variations reflecting a down-regulation of the GTH receptor genes? Gonadal FSHR mRNA expression was found to decrease after exogenous androgen treatment in immature catfish (Schulz *et al.* 2003). We do not exclude that a similar hormonal regulation may occur in trout, since the decrease in relative abundance of rtFSHR transcript also coincides with a slow increase in plasma testosterone levels described previously in our trout strain between stages I and VI (Gomez 1998). However, if we assume that the GTH receptors are mainly expressed in somatic cells, the decrease in the relative abundance of the receptor transcripts would most likely be due to a 'dilution' effect. In fact, when the receptor mRNA abundance is expressed as total amount per testis, no significant decrease in rtLHR or rtFSHR abundance could be detected between stages III and VI of gonadal development. In a recent study, in which the transcript content was expressed per gonad, the amount of FSHR messengers was even found to increase coincidently with gonadal growth and with the increase in germ cell differentiation (meiosis and spermiogenesis; Kusakabe *et al.* 2006). In that study, LHR gene expression also progressively increased as spermatogenesis progressed and was correlated with plasma LH levels. Further studies will be required to determine whether changes in the relative abundance of GTH receptor mRNA only result from changes in the proportion of expressing cells or whether they occur as a result of specific regulations in GTH target cells. Unfortunately, our attempt to locate and quantify trout GTH receptor transcripts using radioactive riboprobes on histological sections failed, most likely because of the low expression levels (Ricordel *et al.*, personal communication).

The expression pattern of trout GTH receptor genes shows a large increase in both receptor transcripts in stage VIII, regardless of the normalization procedure used. This increase

is consistent with studies showing higher LH-binding sites, higher sensitivity to LH in terms of steroid production output (Le Gac & Loir 1988, Planas & Swanson 1995), and high plasma GTH levels (Gomez *et al.* 1999) in prespawning or spawning males. The dramatic increase of rtLHR in stage VIII is also consistent with the fact that, using *in vitro* ligand autoradiography, LH binding could only be detected in spermiating salmon testis (Miwa *et al.* 1994).

To conclude, the present study describes the presence of two distinct GTH receptors in rainbow trout showing similarities with those of higher vertebrates, but also differences in terms of their structural determinants (FSHR). The gonadal expression pattern of the GTH receptor genes suggests that FSHR may play an important role in regulating gonadal functions in spawning trout, in addition to the LHR pathway. *In vitro* functional studies demonstrate that rtLH and rtFSH preferentially activate their cognate receptor.

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References

Basu D & Bhattacharya S 2002 Purification of two types of gonadotropin receptors from carp ovarian follicles: overlapping recognition by two different ligands. *General and Comparative Endocrinology* **129** 152–162.

Billard R & Escafffre AM 1975 Identification des stades de la spermatogenese de la truite fario d'apres la morphologie des gonades et la spermiation. *Bulletin Français de Pisciculture* **256** 111–118.

Bobe J, Maugars G, Nguyen T, Rime H & Jalabert B 2003 Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. *Molecular Reproduction and Development* **66** 46–53.

Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ & Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **64** 1633–1643.

Breton B & Sambroni E 1989 Evolution du nombre des récepteurs gonadotropes ovariens au cours du cycle reproducteur annuel chez la truite fario *Salmon trutta* L. *Comptes Rendus de l'Academie des Sciences. Série III, Sciences de La Vie* **308** 495–500.

Breton B, Le Gac F & Sambroni E 1986 Gonadotropin hormone (GtH) receptors in the ovary of the brown trout *Salmo trutta* L. *in vitro* studies. *General and Comparative Endocrinology* **64** 163–171.

Breton B, Govoroun M & Mikolajczyk T 1998 GTHI, GTH II, secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. *General and Comparative Endocrinology* **111** 38–50.

Campbell B, Dickey J, Beckman B, Young G, Pierce A, Fukada H & Swanson P 2006 Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. *Biology of Reproduction* **75** 34–44.

van Cappellen WA, Meijs-Roelofs HM, Kramer P, van Leeuwen EC, de Leeuw R & de Jong FH 1995 Recombinant FSH(Org32489), induces follicle growth and ovulation in the adult cyclic rat. *Journal of Endocrinology* **144** 39–47.

Fredriksson R, Lagerstrom MC, Lundin LG & Schioth HB 2003 The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology* **63** 1256–1272.

Gabe M 1968 *Techniques Histologiques*., Paris: Masson & Cie p 1113.

Le Gac F & Loir M 1988 Control of testis function in fish: *in vitro* studies of gonadotropic regulation in the trout (*Salmo gairdneri*). *Reproduction, Nutrition, Development* **28** 1031–1046.

Le Gac F, Breton B & Bougoussa M 1988 Gonadotropic hormone (GtH) receptors in the testis of the trout *Salmo gairdneri*: *in vitro* studies. *Fish Physiology and Biochemistry* **5** 209–217.

Garczynski MA & Goetz FW 1997 Molecular characterization of a ribonucleic acid transcript that is highly up-regulated at the time of ovulation in the brook trout (*Salvelinus fontinalis*) ovary. *Biology of Reproduction* **57** 856–864.

Gomez JM. Relations entre croissance somatique et reproduction chez la truite arc-en-ciel (*Oncorhynchus mykiss*): aspects morphologiques et endocriniens au cours de la gamétogenèse. Rennes University. 1998 (Ref. type: Thesis/Dissertation).

Gomez JM, Weil C, Ollitrault M, Le Bail PY & Le Gac F 1999 Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **113** 413–428.

Govoroun MS, Huet JC, Pernollet JC & Breton B 1997 Use of immobilized metal ion affinity chromatography and dye-ligand chromatography for the separation and purification of rainbow trout pituitary gonadotropins, GTH I and GTH II. *Journal of Chromatography. B* **698** 35–46.

Govoroun M, Chyb J & Breton B 1998 Immunological cross-reactivity between rainbow trout GTH I and GTH II and their alpha and beta subunits: application to the development of specific radioimmunoassays. *General and Comparative Endocrinology* **111** 28–37.

Hoak DC & Schwartz NB 1980 Blockade of recruitment of ovarian follicles by suppression of the secondary surge of follicle-stimulating hormone with porcine follicular fluid. *PNAS* **77** 4953–4956.

Jalabert B 1976 *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdnery*), northern pike (*Esox lucius*), and goldfish (*Carassius auratus*). *Journal of the Fisheries Research Board of Canada* **33** 974–988.

Kajava AV 1998 Structural diversity of leucine-rich repeat proteins. *Journal of Molecular Biology* **277** 519–527.

Kajava AV, Vassart G & Wodak SJ 1995 Modeling of the three-dimensional structure of proteins with the typical leucine-rich repeats. *Structure* **3** 867–877.

Kanamori A, Kagawa H & Nagahama Y 1987 Gonadotropin receptors in the postovulatory ovary of amago salmon (*Oncorhynchus rhodurus*). *General and Comparative Endocrinology* **66** 210–217.

Ko H, Park W, Kim DJ, Kobayashi M & Sohn YC 2007 Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies. *Journal of Molecular Endocrinology* **38** 99–111.

Kumar RS, Ijiri S & Trant JM 2001*a* Molecular biology of channel catfish gonadotropin receptors: 1 cloning of a functional luteinizing hormone receptor and preovulatory induction of gene expression. *Biology of Reproduction* **64** 1010–1018.

Kumar RS, Ijiri S & Trant JM 2001*b* Molecular biology of the channel catfish gonadotropin receptors: 2 complementary DNA cloning, functional expression, and seasonal gene expression of the follicle-stimulating hormone receptor. *Biology of Reproduction* **65** 710–717.

Kusakabe M, Nakamura I, Evans J, Swanson P & Young G 2006 Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology* **189** 541–554.

Kwok HF, So WK, Wang Y & Ge W 2005 Zebrafish gonadotropins and their receptors: I cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. *Biology of Reproduction* **72** 1370–1381.

Laan M, Richmond H, He C & Campbell RK 2002 Zebrafish as a model for vertebrate reproduction: characterization of the first functional zebrafish (*Danio rerio*) gonadotropin receptor. *General and Comparative Endocrinology* **125** 349–364.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta$CT method. *Methods* **25** 402–408.

Loir M 1994 *In vitro* approach to the control of spermatogonia proliferation in the trout. *Molecular and Cellular Endocrinology* **102** 141–150.

Loir M 1999 Spermatogonia of rainbow trout: II *in vitro* study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Molecular Reproduction and Development* **53** 434–442.

Maugars G & Schmitz M 2006 Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (*Salmo salar* L). *General and Comparative Endocrinology* **149** 108–117.

Miwa S, Yan L & Swanson P 1994 Localization of two gonadotropin receptors in the salmon gonad by *in vitro* ligand autoradiography. *Biology of Reproduction* **50** 629–642.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H & Nagahama Y 1999*a* Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications* **263** 584–590.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H & Nagahama Y 1999*b* The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications* **265** 366–371.

Oba Y, Hirai T, Yoshiura Y, Kobayashi T & Nagahama Y 2001 Fish gonadotropin and thyrotropin receptors: the evolution of glycoprotein hormone receptors in vertebrates. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **129** 441–448.

Patino R & Sullivan CV 2002 Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry* **26** 57–70.

Planas JV & Swanson P 1995 Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) *in vitro*. *Biology of Reproduction* **52** 697–704.

Planas JV, Athos J, Goetz FW & Swanson P 2000 Regulation of ovarian steroidogenesis *in vitro* by follicle-stimulating hormone and luteinizing hormone during sexual maturation in Salmonid fish. *Biology of Reproduction* **62** 1262–1269.

Prat F, Sumpter JP & Tyler CR 1996 Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biology of Reproduction* **54** 1375–1382.

Quesnel H & Breton B 1993 Solubilization and purification of the gonadotropin (GTH II) receptor from rainbow trout (*Oncorhynchus mykiss*) ovaries. *General and Comparative Endocrinology* **91** 272–280.

Remacle C 1976 The action of hormones on the male germinal cells of *Carassius auratus* L. in organ culture. *General and Comparative Endocrinology* **29** 480–491.

Salmon C, Kagawa H, Adachi S, Nagahama Y & Fontaine YA 1984 Demonstration of specific gonadotropin binding sites of chum salmon (*Oncorhynchus keta*) in membrane preparations of granulosa cells from the ovary of amago salmon (*Oncorhynchus rhodurus*). *Comptes Rendus de l'Acad?mie des Sciences. Série III, Sciences de La Vie* **298** 337–340.

Sambroni E, Gutieres S, Cauty C, Guiguen Y, Breton B & Lareyre JJ 2001 Type II iodothyronine deiodinase is preferentially expressed in rainbow trout (*Oncorhynchus mykiss*) liver and gonads. *Molecular Reproduction and Development* **60** 338–350.

Schulz R, Dijk W & Bogerd J 2003 Sertoli cell proliferation and FSH signalling in African catfish, *Clarias gariepinus*. *Fish Physiology and Biochemistry* **28** 223.

Schulz RW, Menting S, Bogerd J, Franca LR, Vilela DAR & Godinho HP 2005 Sertoli cell proliferation in the adult testis – evidence from two fish species belonging to different orders. *Biology of Reproduction* **73** 891–898.

Sekine S, Saito A, Itoh H, Kawauchi H & Itoh S 1989 Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. *PNAS* **86** 8645–8649.

So WK, Kwok HF & Ge W 2005 Zebrafish gonadotropins and their receptors: II cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits – their spatial-temporal expression patterns and receptor specificity. *Biology of Reproduction* **72** 1382–1396.

Sohn YC, Yoshiura Y, Kobayashi M & Aida K 1999 Seasonal changes in mRNA levels of gonadotropin and thyrotropin subunits in the goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **113** 436–444.

Suzuki K, Kawauchi H & Nagahama Y 1988 Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *General and Comparative Endocrinology* **71** 292–301.

Swanson P, Bernard M, Nozaki M, Suzuki K, Kawauchi H & Dickoff WW 1989 Gonadotropins I and II in juvenile coho salmon. *Fish Physiology and Biochemistry* **7** 169–176.

Swanson P, Suzuki K, Kawauchi H & Dickhoff WW 1991 Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biology of Reproduction* **44** 29–38.

Trinh KY, Wang NC, Hew CL & Crim LW 1986 Molecular cloning and sequencing of salmon gonadotropin beta subunit. *European Journal of Biochemistry* **159** 619–624.

Tyler CR, Sumpter JP, Kawauchi H & Swanson P 1991 Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **84** 291–299.

Tyler CR, Pottinger TG, Coward K, Prat F, Beresford N & Maddix S 1997 Salmonid follicle-stimulating hormone (GtH I) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. *Biology of Reproduction* **57** 1238–1244.

Vischer HF & Bogerd J 2003 Cloning and functional characterization of a gonadal luteinizing hormone receptor complementary DNA from the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **68** 262–271.

Vischer HF, Granneman JC, Linskens MH, Schulz RW & Bogerd J 2003 Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. *Journal of Molecular Endocrinology* **31** 133–140.

Yan L, Swanson P & Dickhoff WW 1992 A two-receptor model for salmon gonadotropins (GTH I and GTH II). *Biology of Reproduction* **47** 418–427.

Zhang R, Buczko E & Dufau ML 1996 Requirement of cysteine residues in exons 1-6 of the extracellular domain of the luteinizing hormone receptor for gonadotropin binding. *Journal of Biological Chemistry* **271** 5755–5760.

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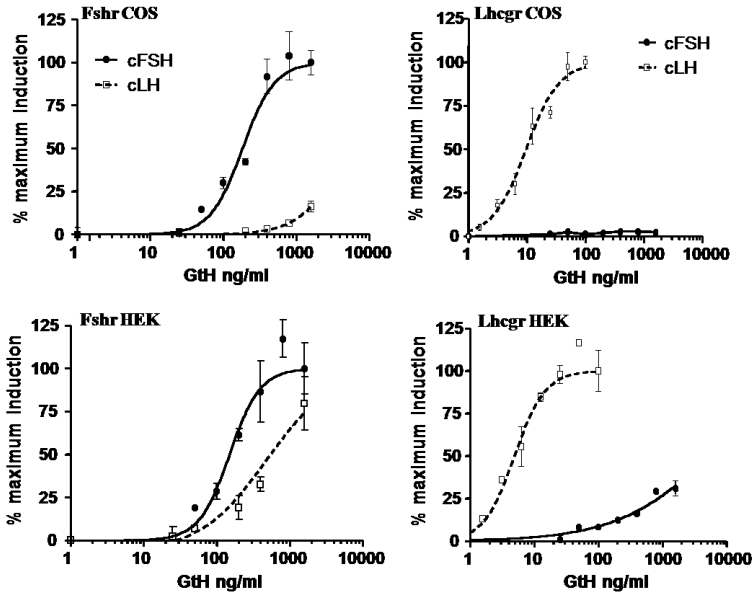

Figure 6 : Expression transitoire des récepteurs Fshr et Lhcgr dans 2 lignées cellulaires mammaliennes (COS-7 et HEK293) et réponses aux gonadotropines de saumon. Les cellules ont été cotransfectées avec le vecteur d'expression exprimant Fshr ou Lhcgr et avec le vecteur rapporteur pCRE-Luc. Les cellules ont été incubées pendant 6 heures avec des doses croissantes d'hormones. La production d'AMPc induite par l'hormone a été quantifiée indirectement en mesurant l'activité de la luciférase du vecteur rapporteur.

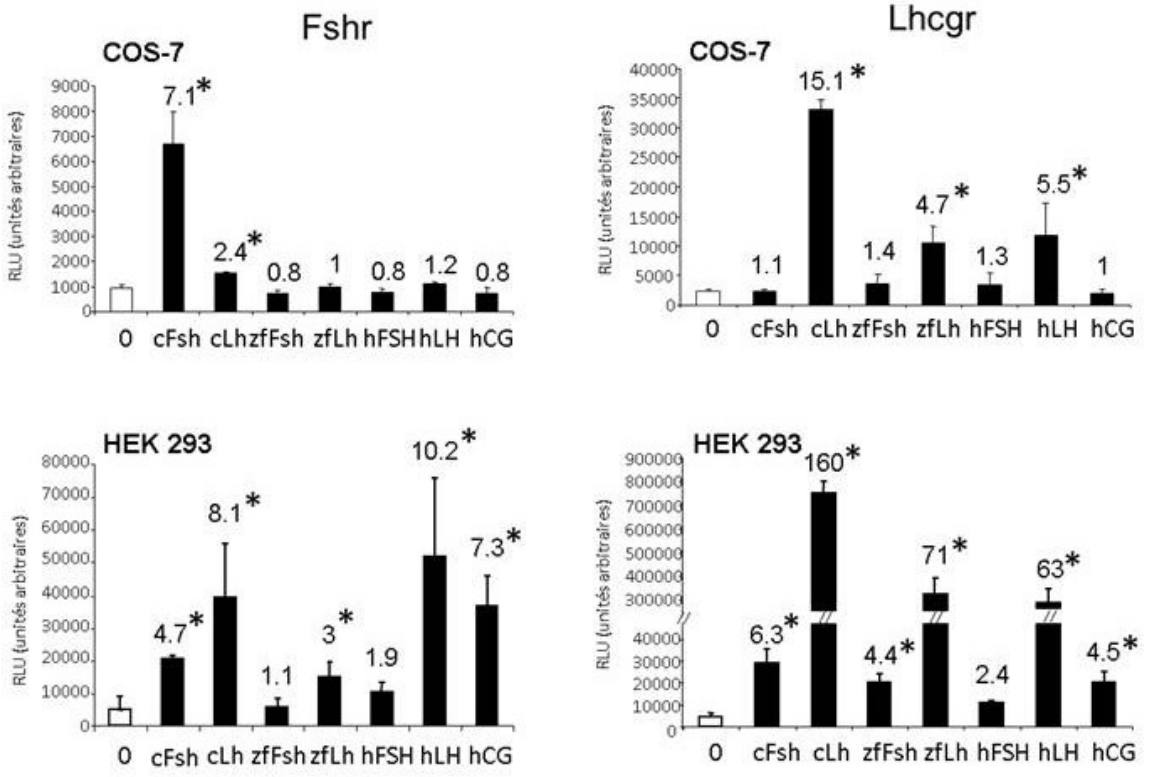

Figure 7 : Comparaison de la réponse des récepteurs Fshr et Lhcgr aux gonadotropines de saumon et aux gonadotropines hétérologues ; cFsh et cLh : Fsh et Lh de saumon chinook, zfFsh et zfLh : Fsh et Lh recombinantes de poisson zèbre, hFSH : FSH humaine, hLH : LH humaine et hCG : gonadotropine chorionique humaine. Les étoiles indiquent une différence de réponse significative entre l'activité luciférase basale et l'activité stimulée par les hormones.

TRAVAUX COMPLEMENTAIRES

Contexte de l'étude

Deux lignées cellulaires sont couramment utilisées pour des études fonctionnelles: COS-7 et HEK 293. En analysant la bibliographie, nous avons constaté que la majorité des travaux décrivant une spécificité non stricte pour les récepteurs de Fsh et de Lh avaient été obtenus en cellules HEK293 et non pas en cellules COS, comme ce fût le cas pour les données publiées dans notre article. Par ailleurs, la sélectivité des récepteurs varie également en fonction des gonadotropines utilisées. Par exemple, chez le poisson zèbre, la Fsh recombinante de l'espèce active uniquement les récepteurs Fshr (So *et al.* 2005), alors que la FSH bovine active les 2 récepteurs, Fshr et Lhcgr (Kwok *et al.* 2005). Des travaux complémentaires ont donc été réalisés chez la truite afin d'examiner d'une part, si la sélectivité des récepteurs des gonadotropines était dépendante du contexte cellulaire et d'autre part, si l'origine et la nature des gonadotropines pouvaient influer sur cette sélectivité. Les récepteurs ont été exprimés dans les 2 lignées cellulaires et leur activation par des gonadotropines issues de salmonidés ou de non salmonidés a été évaluée par la mesure indirecte de la production d'AMPc décrite précédemment (Sambroni *et al.* 2007). Les données d'activation des récepteurs observée dans les deux lignées cellulaires ont été comparées à celles obtenues *ex vivo* sur des cultures d'explants testiculaires et estimées par la capacité des gonadotropines à stimuler la production de 11KT.

Résultats et conclusion

L'expression de Fshr et de Lhcgr dans des cellules COS-7 ou dans des cellules HEK293, toutes choses étant égales par ailleurs, n'aboutit pas aux mêmes résultats de sélectivité. En cellules HEK293, on constate une perte partielle de spécificité des 2 récepteurs vis-à-vis des gonadotropines de saumon chinook (*Oncorhynchus tschawytscha*) (Figure 6), mais aussi vis-à-vis des hormones hétérologues pisciaires (hormones recombinantes de poisson zèbre : zfrFsh et zfrLh) et mammaliennes (hCG, hFSH, hLH) (Figure 7). Les activations par les hormones mammaliennes ne sont pas retrouvées *ex vivo* dans un test d'activité biologique sur la production de stéroïdes par des explants testiculaires en culture. Seules les 2 gonadotropines issues de salmonidés stimulent la production de 11KT alors que les 3 hormones humaines, FSH, LH et hCG, n'ont pas d'effet détectable, même à forte concentration (1.6 µg/mL)(Figure 8).

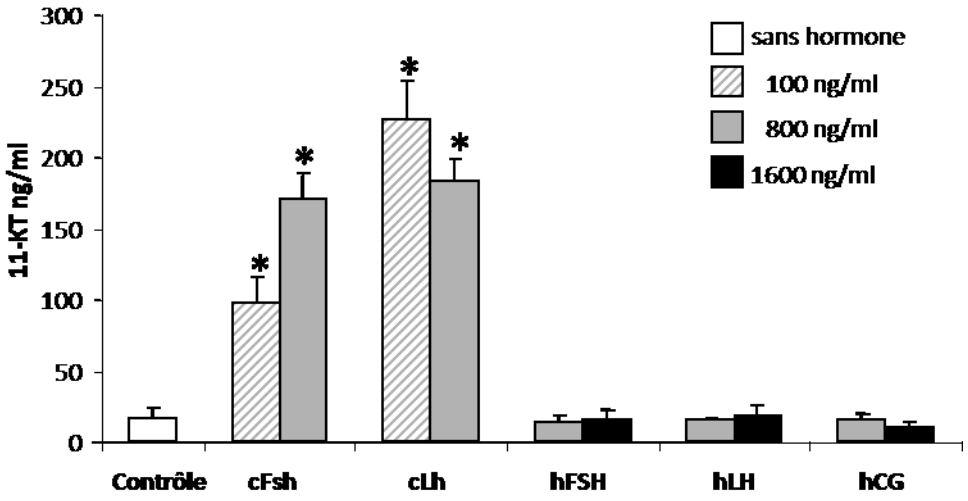

Figure 8 : Production de 11KT par des explants testiculaires incubés pendant 18h en présence de gonadotropines de salmonidés et de gonadotropines mammaliennes. cFsh et cLh : Fsh et Lh de saumon chinook, hFSH : FSH humaine, hLH : LH humaine et hCG : gonadotropine chorionique humaine. Les étoiles indiquent une différence significative entre la production basale de 11KT, sans hormone et la production induite par les hormones (Saha et al., non publié).

Cela montre que le tissu cible se révèle être un environnement favorable à une sélectivité accrue des récepteurs vis-à-vis des hormones mammaliennes. Des études plus approfondies seront nécessaires pour déterminer si cette sélectivité accrue passe par des modifications de glycosylation et/ou de conformation des récepteurs endogènes, Fshr et Lhcgr, qui pourraient affecter la liaison des gonadotropines humaines et/ou la transduction du signal.

Ces travaux complémentaires démontrent clairement que la nature des ligands mais aussi le contexte cellulaire dans lequel les récepteurs sont exprimés influent sur la sélectivité des récepteurs. Ils montrent que, même si la lignée COS-7 offre un environnement cellulaire plus apte à mimer les fonctionnalités des récepteurs observées *ex vivo* que la lignée HEK293, aucune des lignées utilisées ne reflète totalement la sélectivité tissulaire des récepteurs.

Les résultats obtenus incitent à reconsidérer les données bibliographiques quant aux conclusions qui peuvent être déduites à partir des seules études de spécificité réalisées en système cellulaire hétérologue car elles pourraient ne refléter que partiellement la réalité physiologique.

Ces travaux ont été présentés sous la forme d'un poster au 9^{e} Symposium International de Physiologie de la Reproduction des Poissons à Cochin en Inde (9th ISRPF, 9-14 août 2011) (Saha *et al.* 2011).

THE CELL CONTEXT INFLUENCES RAINBOW TROUT GONADOTROPIN RECEPTORS' SELECTIVITY

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Introduction:

Vertebrate reproduction is tightly regulated by glycoprotein hormones produced by the pituitary gland. Two gonadotropins, FSH and LH are present in tetrapod vertebrates and the duality of gonadotropins has become an accepted principle also for fishes, the largest group of vertebrates. The presence of two distinct gonadotropin receptors (GtHRs) in a single fish species was confirmed by the molecular cloning of two different cDNAs in several fish species: salmon, catfish, zebrafish, sea bass, eel and trout. The ligand selectivity of mammalian GtHRs is well defined: FSHR and LHR bind their respective ligands specifically and show little cross-activation (0.01–0.1%). In contrast to the situation in mammalian species, the bioactivity of fish gonadotropins seems to be less well separated as a result of promiscuous hormone–receptor interactions. Depending on the species, hormones, and hormone concentrations used, a promiscuous activation of one or the other fish GtHRs was reported in functional studies using mammalian cell lines expressing fish receptors. In African catfish, recombinant cfFSH and cfLH activated FSHR with a similar biopotency [1] whereas in amago salmon, only FSH was able to activate FSHR. In zebrafish, recombinant zfFSH stimulated only FSHR, whereas recombinant zfLH stimulated both FSHR and LHR [2]. In trout, gonadotropins purified from trout or salmon pituitaries specifically activate their cognate receptor [3]. In summary, data from studies using different bioassays do not allow drawing general conclusions on the responsiveness of the piscine receptors to GtHs. In the present study, we report that the apparent discrepancy in fish gonadotropin receptors cross-selectivity originates mainly in the choice of the cell line used for receptor expression, and also from the heterologous or homologous origin of the hormones tested. The COS-7 cell line led to a highly selective responsiveness of the GtHRs whereas HEK cells show strong cross-reactivity.

Methods:

Rainbow trout FSH and LH receptor expression vectors were constructed using pcDNA3.1/V5-His-TOPO expression vector (Invitrogen) and the GtHR cDNA placed upstream from the polyadenylation site of the bovine growth hormone gene and downstream the cytomegalovirus (CMV) promoter. The HEK293/CREB-Luc cells (Panomics), and COS -7 cells were used for transient transfection assays. HEK293 cells were cultured in presence of 0.2 % hygromycin B (50mg/ml) in the DMEM culture medium in 24 well culture plate at a density of 20000 cell/well for 96 hours. COS-7 cells were cultured in a 24 well culture plate at a density of 70,000 cells/well for 24 hours. After that cells were transfected with either pcDNA3.1/V5-His-FSHR or pcDNA3.1/V5-His-LHR (10ng/well). Cells were stimulated with purified chinook FSH (cFSH), chinook LH(cLH), recombinant zebrafish FSH (zfFSH), recombinant zebrafish LH (zfLH), human FSH (hFSH), human LH (hLH) and human chorionic gonadotropin (hCG) for six hours. Luciferase activity was measured from 40µl lysates using the luciferase assay kit (Promega). Cultures of testicular tissue explants were carried out to analyse the effect of different salmonid and non-salmonid hormones on 11-ketotestosterone (11-KT) production.

Results and discussion:

Functional characteristics of trout GtHRs were analysed in two different cell contexts: COS-7 and HEK-293 cell lines. FSH receptor was efficiently activated by both cFSH and cLH in the HEK293 cell line and similar inductions of FSHR were obtained at 800 ng/ml (maximal fold induction of the luciferase reporter gene was 6.4 with cFSH vs. 6.6 with cLH). The cLH potency appeared to be lower, with an effective half-maximum concentration (EC_{50}) equal to 349 ng/ml versus 111 ng/ml for cFSH. In contrast, in the COS-7 cell line, FSH receptor was only activated by cFSH, with an effect similar to that observed in HEK cells (5.9 fold induction at 800 ng/ml and EC_{50} =133 ng/ml). These results reveal that FSHR response to LH is strongly dependent on the cell context.

LH receptor was mainly stimulated by cLH in both HEK293 and COS-7 cell lines. The maximal fold induction was significantly higher in HEK293 cell compared to COS-7 cell (x79 vs. x14). In addition, the potency of cLH appeared almost twice higher in HEK293 cells than in COS-7 cells, with an EC_{50} value of 5 ng/ml versus 9.8 ng/ml, respectively. This indicates

that HEK cells were more favorable to LH receptor response, as compared to COS cells. Although trout LHR was mainly stimulated by cLH, high doses of cFSH (1600 ng/ml) efficiently activated LHR in the HEK293 cell line but not in the COS cell line. So, our results indicate that the cellular context modulates the selectivity and the amplitude of the LH receptor response.

To address further whether the cell context could modulate ligand-receptor selectivity, we tested the trout GtHRs responsiveness to mammalian and zebrafish gonadotropins. In COS-7 cells, FSH receptor was activated by salmonid FSH but not by the zebrafish or human gonadotropins tested. In HEK cells, FSH receptor was activated not only by salmonid FSH and LH, but also by all heterologous LH and hCG. In COS cells, LH receptor was efficiently stimulated by salmonid LH and moderately by non-salmonid LH but never by any FSH or hCG. On the contrary, in HEK cells, LH receptor was activated by all types of LH and hCG. Interestingly, it was also induced by salmonid and non-salmonid FSH, although to a lower magnitude.

Finally, additional studies revealed that mammalian hormones including hCG, up to 1600 ng/ml, did not induce 11-KT production from rainbow trout testicular explants cultured *ex vivo*.

Conclusions:

We demonstrate that cross-selectivity of the trout GtHRs' responsiveness depends on the cellular context. Trout GtHRs show high ligand selectivity when expressed in COS-7 cells, but not when expressed in HEK cells. Trout receptors selectivity in COS-7 cells seems to reflect better the *ex vivo* conditions. Altogether, we propose that trout GtHRs are highly selective and that the mammalian cell lines used reflect only partially this high selectivity.

References:

[1]BOGERD, J., BLOMENROHR, M., ANDERSSON, E., VAN DER PUTTEN, H.H., TENSEN, C.P., VISCHER, H.F., GRANNEMAN, J.C., JANSSEN-DOMMERHOLT, C., GOOS, H.J., SCHULZ, R.W. 2001. Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biol. Reprod., 64: 1633–1643

[2]KWOK, H.-F., SO, W.-K., WANG, Y., GE, W. 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. Biol. Reprod., 72: 1370–1381.

[3]SAMBRONI, E., LE GAC, F., BRETON, B., LAREYRE, J.J. 2007. Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. J. Endocrinol., 195: 213–228.

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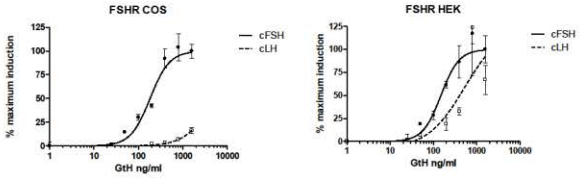
BACKGROUND

The presence of two distinct gonadotropin receptors (GtHRs) in a single fish species was confirmed by the molecular cloning of two different cDNAs in several fish species: salmon, catfish, zebrafish, sea bass, eel and trout. The ligand selectivity of mammalian GtHRs is well defined: FSHR and LHR bind their respective ligands specifically and show little cross-activation (0.01–0.1%). In contrast to the situation in mammalian species, the bioactivity of fish gonadotropins seems to be less well separated as a result of promiscuous hormone–receptor interactions. Depending on the species, hormones, and hormone concentrations used, a promiscuous activation of one or the other fish GtHRs was reported in functional studies using mammalian cell lines expressing fish receptors. In the present study, we report that the apparent discrepancy in fish gonadotropin receptors cross-selectivity originates mainly in the choice of the cell line used for receptor expression, and also from the heterologous or homologous origin of the hormones tested. The COS-7 cell line led to a highly selective responsiveness of the GtHRs whereas HEK cells show strong cross-reactivity.

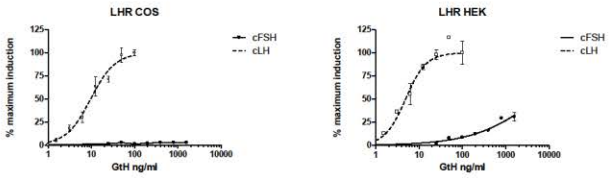
RESULTS

FSH receptor activated by both cFSH and cLH in the HEK293 cell line but only by cFSH in COS-7 cell line



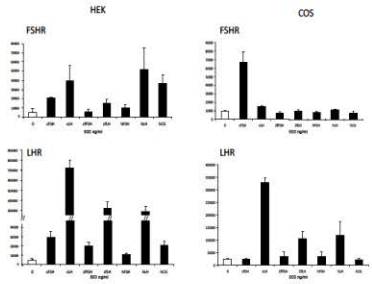
Dose response of cFSH and cLH for rainbow trout FSHR in different cell line

LH receptor mainly stimulated by cLH in both HEK293 and COS-7 cell lines



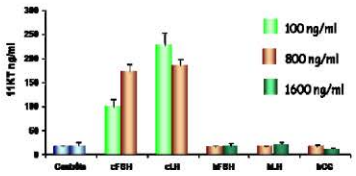
Dose response of cFSH and cLH for rainbow trout LHR in different cell line

Cell context could modulate ligand-receptor selectivity



Transient expression and hormone responsiveness of rainbow trout FSHR and LHR in different cell line.

Hormone effect on testicular steroid production



Effect of different salmonid and non-salmonid hormone on 11-ketotestosterone (11-KT) production

CONCLUSION

We demonstrate that cross-selectivity of the trout GtHRs' responsiveness depends on the cellular context. Trout GtHRs show high ligand selectivity when expressed in COS-7 cells, but not when expressed in HEK cells. Trout receptors selectivity in COS-7 cells seems to reflect better the *ex vivo* conditions. Altogether, we propose that trout GtHRs are highly selective and that the mammalian cell lines used reflect only partially this high selectivity.

Etude du rôle des récepteurs des hormones gonadotropes par immunisation active de la truite au début du cycle gamétogénétique

Chez les poissons, les rôles respectifs des récepteurs Fshr et Lhcgr dans la réponse aux gonadotropines et dans la régulation de la gamétogenèse sont particulièrement peu connus.

Chez la truite mâle, nous avons montré que les ARNm de Fshr et de Lhcgr sont présents à tous les stades de la spermatogenèse et qu'ils présentent une forte élévation pendant la période d'émission des gamètes, période probable d'intense régulation de ces transcrits. Nous avons aussi montré que les récepteurs interagissaient spécifiquement avec leur ligand respectif. L'inhibition spécifique de l'un ou l'autre des récepteurs nous fournirait d'excellents outils pour l'analyse de leurs rôles respectifs dans la spermatogenèse. Notre modèle truite est un bon modèle car nous disposons de populations monosexes mâles, de souche automnale, qui sont élevées de telle sorte que plus de 90 % des animaux initient leur spermatogenèse lors de leur deuxième année, entre février et avril. Nous pouvons donc cibler un stade particulier du cycle pour bloquer l'action des gonadotropines et en évaluer les conséquences sur la progression de la gamétogenèse.

Une méthode de vaccination par "auto-immunisation" contre un fragment peptidique des récepteurs FSHR et LHCGR a été développée et appliquée à différents modèles de mammifères, pré pubères ou adultes (Remy *et al.* 1996; Abdennebi *et al.* 1999; Abdennebi *et al.* 2003). La méthode dérive de la technique de phage display et consiste en une immunisation active contre le phage filamenteux Fd, exprimant à sa surface la protéine p8 (environ 2700 copies par phage) fusionnée avec un décapeptide déduit des séquences des récepteurs. Dix à trente pour cent des protéines p8 à la surface du phage portent alors les 10 acides aminés exogènes. Les décapeptides sont choisis dans la partie N-terminale des récepteurs, plus précisément dans une région de l'exon 1 du gène très spécifique de chaque récepteur, fortement impliquée dans la liaison avec l'hormone et accessible aux anticorps circulants. Une fois injectés dans l'animal, les bactériophages recombinants induisent la production d'anticorps dirigés contre ces épitopes des récepteurs. L'efficacité et la spécificité des anticorps ont été vérifiées à la fois *in vitro* dans des tests de compétition et *in vivo* puisque la vaccination entraîne des perturbations de la reproduction : retard de la maturation sexuelle et du pic pubertaire de testostérone chez le pré pubère (souris et chevreaux ;

(Abdennebi *et al.* 2003) et inhibition de la production de spermatozoïdes sans modifier les niveaux circulants d'androgènes chez le singe adulte (Rao *et al.* 2004). Nous avons appliqué cette méthode de vaccination chez la truite et cherché à vérifier si l'inhibition de la réceptivité à Fsh et/ou à Lh -si elle est effective- se traduit par une perturbation de la spermatogenèse à une étape donnée de ce processus (prolifération des cellules germinales, différenciation des gamètes).

En résumé, nous montrons chez la truite que la vaccination contre les récepteurs des gonadotropines a des effets inhibiteurs sur les fonctions gonadiques observables chez les mâles et chez les femelles. Les 2 fonctions gonadiques (gamétogenèse et stéroïdogenèse) sont touchées. Chez le mâle vacciné contre les 2 récepteurs, on observe une diminution significative du RGS et un retard de la spermatogenèse, visible par une réduction des stades post méiotiques. La vaccination a un effet global inhibiteur sur la production de T, 11KT et E2. On peut observer des effets différents pour les vaccins antiFSHR et antiLHR démontrant qu'on est capable d'inhiber spécifiquement l'un ou l'autre des récepteurs.

Ce travail a été effectué dans le cadre d'une collaboration avec nos collègues Jean-Jacques Remy et Latifa Abdennebi-Najar. Il a été publié dans General and Comparative Endocrinology sous le titre « Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Oncorhynchus mykiss* » (Sambroni *et al.* 2009). Les résultats ont aussi été présentés sous forme d'un poster au congrès « 8th International Symposium on Reproductive Physiology of Fish » (ISRPF) qui s'est tenu à St Malo en juin 2007 et sous forme de communications orales dans le cadre de séminaires locaux (IFR et PEIMA).

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Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

In fish, gonadotropin hormones FSH-GTH1 and LH-GTH2 are less specific for their cognate receptors than in mammals. The respective reproductive functions of fish LH and FSH are thus difficult to establish. We aimed to study the effect of specific antagonists of the two gonadotropin receptors on trout sexual maturation in both sexes by targeting specific regions of LH and FSH receptors, Lhr and Fshr. Filamentous phages displaying Lhr specific or Fshr specific decapeptides from the extracellular hormone binding domain were engineered. Recombinant phages were used as receptor-specific antagonistic vaccines. Male and female trouts were immunized with anti-LHR, anti-FSHR, anti-FSHR + LHR or adjuvant alone, through multiple injections over 8–24 weeks, starting at different stages of sexual maturation. The consequences of immunization on gonadal development were evaluated by determining gonad growth, by histological analysis of testis and ovaries at the end of the vaccination period and by measuring blood plasma sex steroids using radioimmunoassay.

We show for the first time in fish that the anti-receptor vaccinations could have specific antagonistic effects on the development of the reproductive functions; while the anti-FSHR affected the sexual maturation of prepubertal males and delayed sperm production, the anti-LHR blocked vitellogenesis in females. In maturing males, the combined anti-FSHR + LHR vaccine inhibited spermatogenesis and affected steroidogenesis. In that case, the effects of the vaccine on spermatogenesis were transient and reversible when immunization was stopped. Such an immunological strategy to specifically and transiently inhibit a receptor provides a promising approach for discovering their specific functions; it could also lead to a new technology for controlling the onset of puberty in aquaculture species.

1. Introduction

In fish, as in mammals, reproductive functions are triggered by the action of two pituitary gonadotropins, via their binding to G protein-coupled receptors in the gonad (Schulz et al., 2001; Mateos et al., 2002). The two receptor genes are thought to have evolved from one single GTH receptor gene during vertebrate evolution. In salmonids, numerous data have been gathered on gonadotropin expression and secretion profiles (Weil et al., 1995; Breton et al., 1998; Gomez et al., 1999), on FSH and LH receptor expression profiles (Miwa et al., 1994; Campbell et al., 2006; Kusakabe et al., 2006) and on the effects of *in vitro* and *in vivo* hormonal treatments (Nagahama et al., 1994; Tyler et al., 1997; Ko et al., 2007). These data suggest that FSH may be responsible for early gametogenesis and gonadal growth control whereas LH would regulate final gonadal maturation and spermiation/ovulation. However, both gonadotropins play a prominent role in the regulation of steroidogenesis, with FSH and LH sharing a similar spectrum of steroidogenic activities, and LH being more potent than FSH in stimulating steroidogenesis in the late stages of gonad development (Schulz et al., 1992; Planas et al., 1993, 2000). Another characteristic of piscine gonadotropins relies on their specificity towards their cognate receptors. Whereas mammalian FSH and LH are highly specific for their cognate receptors with less than 0.1% cross activation (Tilly et al., 1992), in many fish species from different orders LH has been shown to bind to or to activate Fshr (Yan et al., 1992; Kumar et al., 2001; Vischer et al., 2003; So et al., 2005; Kazeto et al., 2008). Furthermore, in amago salmon and rainbow trout, while each hormone mainly activated its cognate receptor, high FSH concentrations were able to activate Lhr (Oba et al., 1999; Sambroni et al., 2007). Thus, in fish, the delineation of the biological functions of each gonadotropin remains unclear. Moreover, little is known about gonadotropic effects on gonad or germ cell development, which would not be mediated by steroids (Schulz and Miura, 2002).

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New approaches targeting gonadotropin receptors could be useful to elucidate their physiological roles in trout gametogenesis and could be helpful for the control of sexual maturation in aquaculture species.

In mammals, we have demonstrated in previous studies that targeting specific regions of LH and FSH receptors through the immune pathway induces impairment of adult fertility (Remy et al., 1996; Abdennebi et al., 1999; Rao et al., 2004) and delays sexual maturity (Abdennebi et al., 2003). In the present study, we used the same phage display strategy to express multiple copies of rainbow trout FSH and LH receptor epitopes on the surface of phage particles. Once injected into fish, the recombinant bacteriophages will induce the production of antibodies against those receptor-specific epitopes. The anti-FSHR and anti-LHR vaccines used in this study were designed before the publication of the 3D structure of the human FSH/Fshr complex (Fan and Hendrickson, 2005). Primary sequence alignments and secondary structure predictions (Sambroni et al., 2007) suggest that the overall spatial structures of the gonadotropin receptors are conserved between mammals and trout. We chose to immunize against N-terminal amino acids because the receptors N-termini (i) share poor homology between Fshr and Lhr (ii) are not buried into the horseshoe-like structure, and (iii) may interact with the specific beta subunit of the hormones.

We aimed to study the effect of immunization against Lhr and Fshr or against both receptors on sexual maturation in the rainbow trout. The effects of anti-receptor vaccines on gametogenesis and on plasma steroids were evaluated in male and female rainbow trout. For the first time this strategy allowed the effect of anti-gonadotropin receptor immunization on the control of reproduction in fish to be investigated.

2. Materials and methods

2.1. Construction of recombinant phages displaying FSH and LH receptor peptides (Genbank Accession No.: AF439405 and AF439404, respectively)

Filamentous phages displaying decapeptides of the rainbow trout FSH receptor and of the LH receptor were engineered to be used as peptide vaccines. Four pairs of complementary oligonucleotides with compatible PstI and HindIII end regions were purchased from EUROGENTEC (France). Two pairs encoded two different overlapping decapeptides of the trout FSH receptor N-terminus, respectively, Fshr-A peptide NTITHMPTHI, and Fshr-B peptide THIPKNTTDL; and two others encoded two overlapping trout LH receptor N-terminus peptides, respectively, Lhr-C NNITEKSVPT, and Lhr-D VPTSERGPRL (Fig. 1). Annealed oligonucleotides were ligated to the double stranded replicative-form DNA of PstI–HindIII restricted phage f88-4. The transformation of female MC1061 *Escherichia coli* yielded tetracycline-resistant colonies. Expected hybrid p8 protein primary structures were verified by sequencing the recombinant DNA in phages Fshr-A, Fshr-B, Lhr-C and Lhr-D with a specific p8 primer using the automated DNA sequencer ABI 310 (PE Biosystems, Courtaboeuf, France).

The production and purification of recombinant phages were carried out as described previously (Abdennebi et al., 2003). After suspension in phosphate saline buffer (PBS, 50 mM, pH 7.4, phosphate, 150 mM NaCl) at a concentration of 100 mg/ml, the phages were inactivated by heating at 70 °C for 15 min and by UV exposure. These were then kept at −20 °C until use for vaccination.

2.2. Animals, immunization protocols and sampling

Experiments were carried out on rainbow trout *Oncorhynchus mykiss* reared at the PEIMA experimental fish farm (INRA, Sizun, Finistère, France). We used two different populations: (1) an all-male population for Experiments I and II, reared in such conditions to obtain more than 95% of males maturing at 2 years old, and (2) a normal population (bisexual) in Experiment III. As a consequence of different rearing conditions, the populations had different body weight at the same age.

Besides, we aimed at investigating the influence of the reproductive stage on the response to immunisation. Immunization procedure started either before (December for Experiment I) or after (April for Experiments II and III) trout reproductive maturation had started.

In Experiments II and III, the boost injections were more frequent to potentially induce a stronger response than in Experiment I.

All fish handling was done on anesthetized animals (phenoxyethanol 0.3 ml/L). All fish were individually tagged 2 weeks before the beginning of experiment.

2.2.1. Experiment I

In December, 1-year-old immature males (body mean weight 127 g) were separated into 4 groups of 25 individuals and injected intraperitoneally with the different vaccines once a month, for 6 months. The treatments were as follows: (1) Adjuvant group: 0.1 ml of PBS + 0.1 ml of a water/oil adjuvant, Montanide ISA50 V2 (SEPPIC, Paris, France), (2) Anti-FSHR group: pool of Fshr-A and -B phages (0.25 mg of each phage diluted in 0.1 ml of PBS + 0.1 ml of adjuvant), (3) Anti-LHR group: pool of Lhr-C and -D phages (0.25 mg of each phage diluted in 0.1 ml of PBS + 0.1 ml of adjuvant), (4) Anti-FSHR + LHR group: mixture of all LHR and FSHR phages, (0.125 mg of each in 0.1 ml of PBS + 0.1 ml of adjuvant; only half the dose of each construct was injected so that fish received the same amount of recombinant phage as in other groups). Each group was reared in 2-m diameter circular tanks, with identical water supply and external conditions (constant water temperature of 10 °C).

Blood sampling was performed at specific times during the experiment for evaluation of the immune responses and for measurement of blood testosterone and 11keto-testosterone. At the end of the experiment (June), we checked for sperm production and gonads were weighed to evaluate sexual maturation.

```
                                                    A          B
trout FSHR MMKMKKIMKM LLCMLGCVCV SQAEVAMVNS GTTFTYLCMG NTITHMPTHI PKNTTDLEFK QTHIRVFPRE AFT------ 73
human FSHR -MALLLVSLL AFLSLGSGCH HR----ICHC SNR-VFLCQE SKVTEIPSDL PRNAIELRFV LTKLRVIQKG AFSGFGDLE 73

                                                        C          D
 trout LHR  -MMSISLLFL FYPYVLLFFG FGCGYTSSFV CPGICRCSAN -TIRCNNITE KSVPTSERG- PRLVLKHLTM  67
 human LHR  MKQRFSALQL LKLLLLLQPP LPRALR-EAL CPEPCNCVPD GALRC----- ---PGPTAGL TRLSLAYLPV  61
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Fig. 1. Alignments of the N-terminal sequences of rainbow trout and human FSH and LH receptors (Genbank Accession Nos.: AF439405 and AF439404, for rainbow trout Fshr and Lhr, respectively, and AAA52477 and AAB19917 for human Fshr and Lhr, respectively). The two overlapping decapeptides of the trout FSH receptor or LH receptor displayed on the recombinant phages are indicated in bold and highlighted with a black line.

2.2.2. Experiment II

In April, male trout in their second year (body mean weight 350 g) were separated into 4 groups of 20 individuals and injected intraperitoneally every 2 weeks for 8 weeks. The four groups were as follows: (1) Adjuvant group: 0.25 ml of PBS + 0.25 ml of an experimental adjuvant GERBU≪734≫ (GERBU Biotechnik GmbH, Am Kirchwald, Germany), (2) Anti-FSHR group: pool of the Fshr-A and -B phages (0.5 mg of each phage diluted in 0.25 ml of PBS + 0.25 ml of adjuvant), (3) Anti-LHR group: pool of the Lhr-C and -D phages (0.5 mg of each phage diluted in 0.25 ml of PBS + 0.25 ml of adjuvant), (4) Anti-FSHR + LHR group: mixture of all LHR and FSHR phages, (0.25 mg of each in 0.25 ml of PBS + 0.25 ml of adjuvant). Groups 1 and 4 on one side, and 2 and 3 on the other side, were reared in the same circular tanks of 2-m diameter (water temperature from 9.6 to 11.8 °C).

2.2.3. Experiment III

To investigate the vaccines' effect on ovarian development, a protocol similar to the one described for Experiment II was applied to another batch of fish containing males and females, the sex was not distinguishable at this stage of development (body mean weight 700 g, 15 fish per group). In this case, fish were immunized with the adjuvant, the anti-FSHR and the anti-LHR vaccines. Only females were analyzed at the end of the experiment. All groups were maintained in the same circular 4-m diameter tank.

In Experiments II and III, vaccine injections and tissue samplings were performed following the schedule reported in Fig. 2. Plasma sex steroid concentrations were measured for 10 fish per group. Male and female gonad development was analyzed by measuring the gonad weight and by histological analysis of gametogenesis 12 days after the last injection. Furthermore, in Experiment II, 10 males per group were kept for gonad examination 10 weeks after the last injection.

2.3. Immune response

The kinetics and intensity of immune response to vaccination were evaluated by ELISA against phages. Microplates were coated with solubilized phages in 100 µl of coating buffer (50 mM $NaCO_3$, pH 8.8) overnight at 4 °C and blocked in PBS (pH 7.4) with 1% BSA (Sigma, St. Quentin Fallavier, France) to avoid non-specific binding. Sera from experimental animals diluted at 1/400 in PBS with 1% BSA, were added to duplicate wells and then incubated for 1 h at 37 °C. Wells were washed twice with phosphate buffer, and then incubated for 2 h at 37 °C with rabbit anti-salmonid Ig antibody (Serotec, France) at a dilution of 1/5000. After 3 times washing, 100 µl of biotynilated sheep anti-rabbit IgG (Serotec, France), complexed to streptavidin peroxidase, (Amersham Bioscience, Uppsala, Sweden) was added to each well at a dilution of 1/10,000, and the plates were incubated for 1 h at 37 °C. The TMB Kit (KPL, Maryland, USA) was used as the peroxidase substrate. Optical densities at 450 nm were then determined.

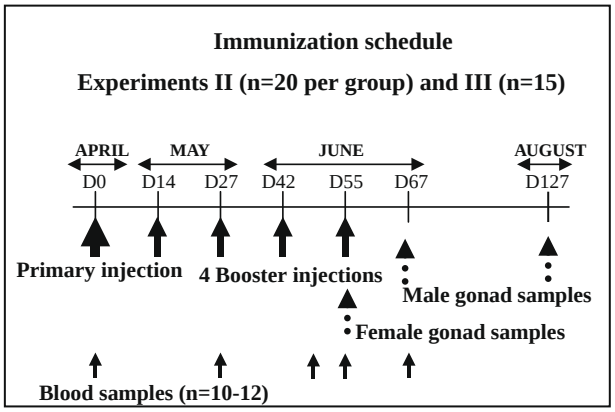

Fig. 2. Immunization protocol in Experiments II and III. Primary and booster immunizations were performed every 2 weeks as indicated by large arrows, over a period of 8 weeks. Thin arrows indicate the time of blood sampling.

2.4. Gonadal stage determination

In fish, sexual maturation is usually evaluated by measuring the gonadosomatic index (GSI = testicular or ovarian weight × 100/body weight). At tissue sampling times, the GSI was determined and a transversal section from the middle part of the gonad was fixed in Bouin solution for histological examination. Fixed gonads were dehydrated and embedded in paraffin, and 5 µm sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue.

The maturity stages of the gonads were evaluated based on the presence and on the relative abundance of the most developed germ cells, according to a classification described by Gomez et al. (1999). In the autumnal spawning strain, most animals remain sexually immature (stage I, GSI = 0.06 ± 0.01%) during 1 year, and active gametogenesis starts in February–March of their second year. In the male, it is indicated by the presence of proliferating spermatogonia (stage II) and the appearance of early meiotic cells (stage IIIa). The first round spermatids appear in stage IIIb and first spermatozoa in stage IV. Testicular weight remains low during stages II–IIIb of spermatogenesis, and then increases dramatically between stage IV (GSI = 0.30 ± 0.03%) and VII (GSI = 5.5 ± 0.3%), when a rising number of cysts are going through meiosis and complete spermiogenesis. In the female, early vitellogenic events were assessed by histological observation of the apparition of cortical alveoli (stage 2), lipidic globules (stage 3), or yolk globules [stages 4.1: first apparition of yolk globules (yolk proteins storage) in a few oocytes; 4.2–4.5: increasing number and size of yolk globules]. Ovarian growth is slow in the stages of pre-vitellogenesis to early vitellogenesis (2–4.2), and then increases dramatically towards the end of vitellogenesis (stages 4.3–4.5).

2.5. Blood plasma sex steroid concentrations

To assess the effect of anti-LHR and anti-FSHR immunizations on the steroidogenic function of the gonads, plasma steroid levels were measured in blood at different times during the immunization procedures. T, 11keto-testosterone (11KT) or estradiol (E2) were measured by specific radioimmunoassay (RIA) following extraction with cyclohexane/ethyl acetate (50:50, v/v), according to Fostier et al. (1982). The [^{3}H]-tracers and the unlabelled hormone came from Amersham Biosciences (GE Healthcare Europe GmbH, Orsay, France). The assay sensitivity was 25 pg/ml.

2.6. Specific growth rate

To evaluate the effects of the vaccines on the growth of fish, the specific growth rate was calculated according the following formula: $\mathrm{SGR} = \frac{\mathrm{Ln}M_f - \mathrm{Ln}M_i}{t} \times 100$ where t represents the period in days, Ln the Neperian logarithm, M_i and M_f the initial and the final body weight, respectively.

2.7. Immunoglobulin purification and action on gonadotropin receptor transactivation

We purified IgM from trout sera using the IgM Purification Kit from Pierce (Rockford, IL) based on affinity exchange on Mannan Binding Protein (MBP) covalently attached to an agarose support, according to the manufacturer's instructions. We used pools of

two sera obtained 10 days after the last boost injection from control, anti-LHR and anti-FSHR vaccination groups (Experiment I). For each pool four runs of purification procedures were conducted resulting in about 5 mg of purified IgM per group.

COS-7 cells were transiently co-transfected with the trout Fshr and a cAMP-responsive reporter construct pCRE-Luc (Stratagene, La Jolla, CA) and cultured in the presence of trout FSH. FSH receptor activation was measured through the luciferase activity, as previously described in Sambroni et al. (2007) with the following changes: COS-7 cells were seeded in Petri dishes Ø10 (10 ml of culture medium) and co-transfected with 500 ng of the gonadotropin receptor expression vector and 5 μg of the cAMP-responsive reporter construct pCRE-Luc. Four hours after transfection, cells were trypsinized and reseeded in 48-wells plates overnight. Twenty-four hours after transfection, cells were pre-incubated for 2 h in the presence or absence of one of the IgM preparations at 2 mg/ml, before adding FSH (500 ng/ml) for another 6-h incubation. Luciferase activity was measured from 40 μl of the lysates using the Luciferase Assay Kit (Promega, Madison, WI). FSH alone induced a 7-fold stimulation of the luciferase reporter gene expression.

2.8. Statistical analysis

Data were analyzed by using Statistica software (Statsoft, France). For each date, the non-parametric test Kruskal–Wallis was used to assess the global effect of treatment. Paired comparisons were then performed for data with a significant ($p < 0.05$) main effect using the Mann–Whitney U test. The values are expressed as mean ± SEM. In Experiment II gonadal and body development were not found to be different between untouched controls and fish adjuvant injected controls; these groups were pooled before further statistical analysis.

Stages of gametogenesis reached at the sampling dates were expressed as a % of the total number of fish in each group. Changes in proportion were analyzed using either the Fisher's exact test or the Taillard's test (Taillard Éric et al., 2008).

Ethics Investigations were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

3. Results

3.1. Immune response

The dynamics of the trout immune response were evaluated by monitoring the humoral response directed against phages by specific ELISA at different times during the course of vaccination in Experiment I. The monitored responses to the three vaccines, relatively slow and moderate, kept increasing up to day 72 (i.e. 1 month after the second booster injection) then tended to level off until the end of the immunization period (Fig. 3).

3.2. Gonadotropin receptor vaccines effects on gonad development

3.2.1. Experiment I

In Experiment I, the vaccination procedure was started in December while all males were in prepubertal stage I. At the end of the experiment (June), gonads were weighed and sperm production was checked to evaluate testis maturation. All males in the adjuvant group and in the LHR vaccine group had reached late stages of gonad development, based on GSI, and milt release could be observed in one third of the fish. Conversely, spermiation was significantly delayed in the anti-FSHR groups (anti-FSHR alone: 5% of the mature fish giving sperm (1/20); anti-FSHR + LHR: 9.1%, (2/23) as compared to adjuvant or anti-LHR groups (29%; $p < 0.05$) (Fig. 4A). Moreover, in the anti-FSHR vaccine group 3 animals out of 23 (13% against 0% in controls; $p < 0.05$) remained at immature stage I, linked to a reduced mean GSI, suggesting a complete inhibition of sexual maturation in these animals (Table 1, Experiment I). In a complementary experiment, we verified that only the vaccination with phage displaying a gonadotropin receptor peptide had inhibitory effects on trout spermatogenesis, while phage displaying random peptides had no significant effect (Table 1, complementary experiment).

In the adjuvant group, the plasma testosterone and 11KT increased as expected in the last blood samplings corresponding to late stages of male maturation. The three anti-gonadotropin receptor vaccinations tended to inhibit the androgen circulating levels. A statistically significant decrease was obtained with the anti-FSHR

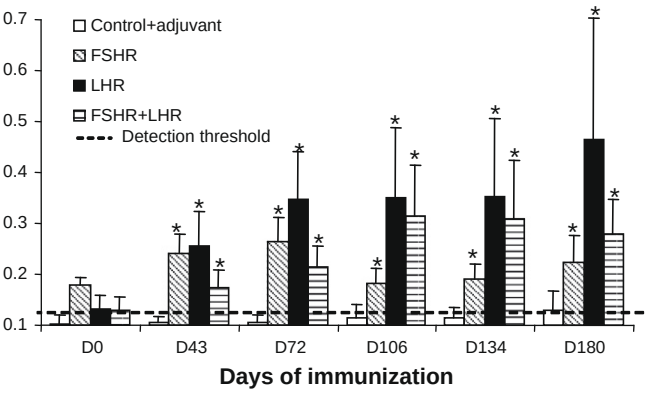

Fig. 3. Immune responses (Ig) to vaccination against phages displaying gonadotropin receptor peptides. Blood sera of sampled fish were diluted to 1/400 and the relative level of the antibody titer against phage was determined by ELISA. The "Control + Adjuvant group" corresponds to data from control fish (no injection) and from the adjuvant treated group. Data are expressed as mean ± SD ($n = 8$–12 individuals). The dotted line represents the threshold for specific Ig detection. *Significant different from control group ($p < 0.01$).

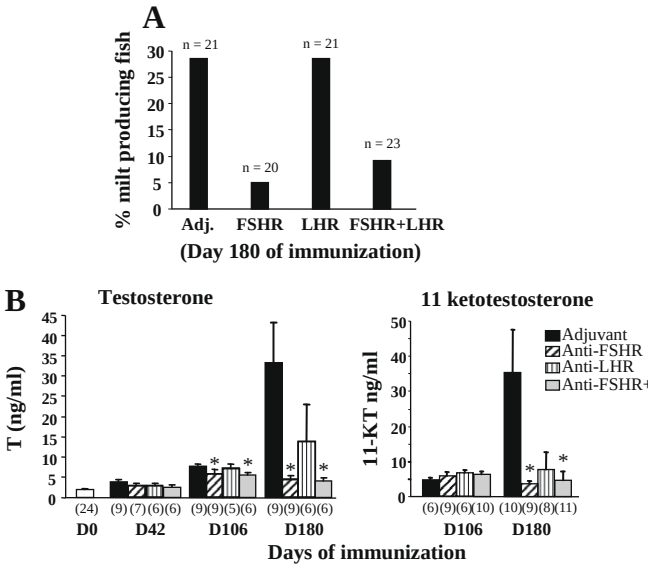

Fig. 4. Vaccination effects on gonadal function in immature male fish. (A) Spermiation was delayed in males immunized against FSHR. The figure shows the proportion of fish producing milt at the end of Experiment I, expressed in % of maturing fish in each group. (B) Blood plasma testosterone and 11KT concentrations (mean ± SEM) at different sampling times in males of Experiment I (n is indicated under the bars). *Significant paired comparison with adjuvant group.

Table 1
Effect of immunization on sexual maturation in Experiment I and complementary experiment.

Group	Immature	Mature (VI and VII)	GSI mean ± SEM
Experiment 1			
Adjuvant	0	21	2.922 ± 0.291
Anti-FSHR	3*	20	2.306 ± 0.293
Anti-LHR	0	21	3.303 ± 0.307
Anti-FSHR + LHR	0	23	2.745 ± 0.197

Group	Immature	IIIa	IIIb	IV	V and VI	(No. of total)
Complementary experiment						
Adjuvant	1	1	4	10	11	(27)
Random peptide phage	2	1	1	18	8	(30)
Anti-FSHR + LHR	6**	0	5	12	7	(30)

* Significantly different from "Adjuvant" and from "anti-LHR" groups ($p < 0.05$).
** Significantly different from "Adjuvant" and "random peptide phage" groups ($p < 0.05$).

vaccine alone, or in combination with the anti-LHR vaccine, for testosterone from day 106, and for 11KT on the last sampling date ($p < 0.05$ compared to adjuvant alone, Fig. 4B).

3.2.2. Experiment II

In Experiment II, the vaccination procedure commenced in pubertal males showing heterogeneous maturation stages (D0, April, Fig. 5B). Between April and June, the mean GSI progressed (from 0.2% to 1%) as expected in the adjuvant group in relation to spermatogenetic development (Fig. 5A). At the end of the immunization period, as compared to the control, there was a significant treatment effect ($p < 0.05$); the mean GSI was lower in males immunized against LHR alone or against the combination of anti-FSHR + LHR. Furthermore, although all fish received similar amounts of phage vaccine, GSI was significantly lower in the anti-FSHR + LHR group when compared to the anti-FSHR group, suggesting a specific effect of the anti-LHR vaccine.

The spermatogenetic process was inhibited by the anti-FSHR + LHR vaccine as observed by histological analysis of the gonads (Fig. 5B): While 80–90% of the males had reached stages IV and V (initiation of spermiogenesis and appearance of spermatozoa) in the other groups, only 40% had reached that stage in the anti-FSHR + LHR immunized fish ($p < 0.05$, Taillard's test). More strikingly, in this group 30% of the gonads presented mainly early spermatogonia (stages II and IIIa) against 0% in the adjuvant or anti-FSHR groups ($p < 0.05$, Taillard's test). The FSHR vaccine alone had no detectable effect. Histological changes are illustrated in Fig. 7e–h.

In August, 10 weeks after the vaccine treatment was stopped, inhibitory effects of immunization were no longer observed, and in all groups the fish had reached the pre-spermiation or spermiation stages (VI and VII), indicating a full reversibility of the method (data not shown).

Blood testosterone increased as expected between April and June. Some variability of this parameter was observed in all groups, linked to the fact that individual fish are not synchronous in their gonadal development. Anti-FSHR or anti-LHR immunization induced a decrease in testosterone plasma levels. As shown in Fig. 5C, statistical analysis by date indicated a significant inhibition only at day 49 for the three vaccines ($p < 0.05$), more pronounced in the anti-FSHR + LHR group ($p < 0.01$). It is noticeable that this sampling date is the only one that took place 7 days after the previous booster injection (instead of after about 15 days for other samples).

3.2.3. Experiment III

In this experiment, female trout in their second year were immunized with the LHR and FSHR vaccines. As for males in

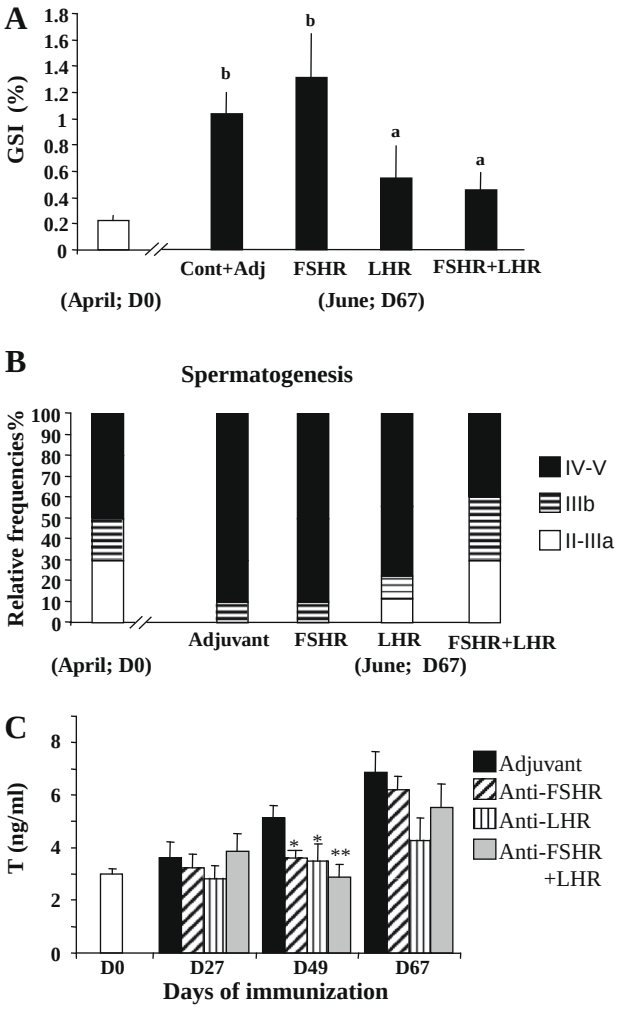

Fig. 5. Vaccination effects on gonadal function in pubertal male fish. (A) GSI at the beginning of the experiment (D0), and in June, 12 days after the last vaccine injection (mean ± SEM). Bars sharing the same letters are not statistically different. (B) Spermatogenetic stages determined by histological study as described in Section 2, and expressed as a % of total fish in each group (indicated in brackets on the bars). II and IIIa: first occurrence of B spermatogonia and early meiosis; IIIb: meiosis; IV and V: first occurrence of spermiogenesis. (C) Blood plasma testosterone concentration (mean ± SEM) at different sampling times in 10 males of Experiment II. *Significant paired comparison with adjuvant group ($p < 0.05$), **significant paired comparison with adjuvant group ($p < 0.01$).

Experiment II, they exhibited variable gonad maturation stages at day 0 of the experiment (Fig. 6B). Vaccine effects on ovarian development were assessed by histological observation of the appearance of the different vitellogenic events. Twelve days after the last injection in June the vitellogenesis process appeared inhibited in immunized females, with each of the two vaccines. The anti-LHR vaccine (Fig. 6B) was efficient enough to maintain mean ovarian development at the stages observed in the preimmunized animals in April. While in the adjuvant group 75% of the females had reached stage 4 of vitellogenesis, only 40% had reached that stage in the anti-FSHR immunized group ($p = 0.134$, Fisher's test) and only 25% in the anti-LHR group ($p < 0.05$, Fisher's test). At that stage, the ovarian development delay induced by the vaccines had little consequence on mean gonad weight or GSI (Fig. 6A). Histological differences are illustrated in Fig. 7a–c.

Both vaccines inhibited plasma testosterone concentration as early as 27 days after the beginning of immunization, although inhibition was statistically significant only at days 49 and 55 of immunization. In paired comparisons, inhibition was more

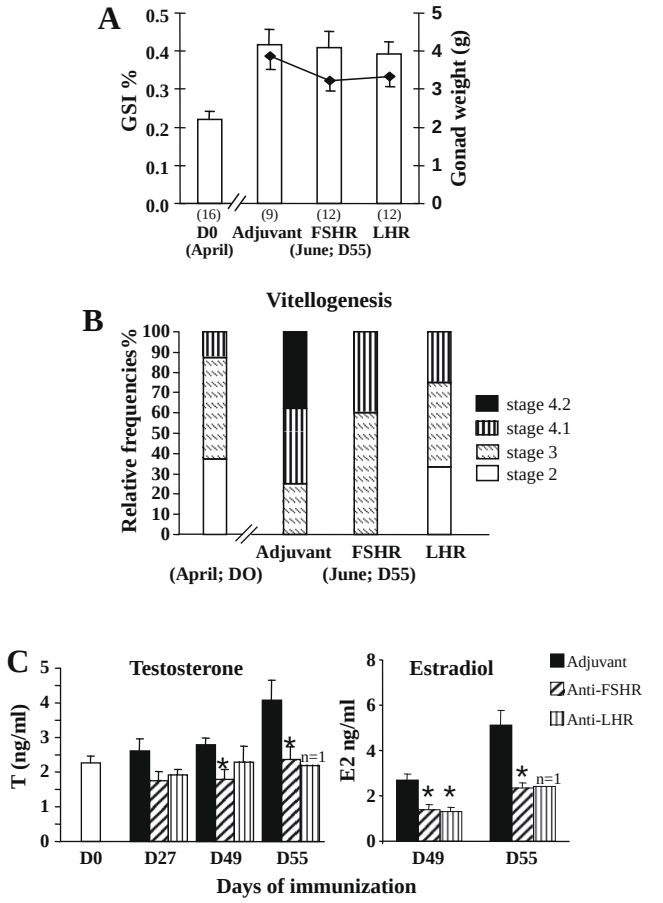

Fig. 6. Vaccination effects on ovarian function in pubertal female trout. (A) GSI and gonad weight determined at the beginning of the experiment (D0), and in June, 12 days after the last booster injection. Each bar and curve point represent the mean ± SEM of *n* individuals (indicated in brackets under the bars). (B) Stages of vitellogenesis development determined by histological study and expressed as a % of total fish in each group. Stage 2: presence of cortical alveoli; stage 3: first lipid globules; stages 4.1 and 4.2: first apparition of yolk globules. (C) Blood plasma testosterone and estradiol concentrations (mean ± SEM) at different sampling times in the groups ($n = 8–12$ except at D55 for the anti-LHR group, $n = 1$. *Significant paired comparison with adjuvant group.

pronounced in the anti-FSHR group ($p < 0.05$). E2 plasma concentrations were also measured at the two last sampling times and show similar inhibitory effects of the two vaccines (Fig. 6C).

In all groups of Experiments II and III (as well as in the complementary experiment reported in Table 1), some male and female gonads showed disorganized tissues in the peripheral areas (Fig. 7d and h) suggesting a possible inflammatory reaction due to the effect of the adjuvant.

3.3. Are the biological effects due to respective antibody for the receptors?

In fish, IgM being the major immunoglobulins involved in immune response, we purified IgM from sera of immunized or control fish. Then we tested these preparations on COS-7 cells co-transfected with the trout Fshr and a cAMP-responsive reporter construct pCRE-Luc and cultured in the presence of an optimum dose of FSH. In this experiment, FSH (500 ng/ml) induced a 7-fold stimulation of the reporter gene expression (Fig. 8). IgM prepared from control serum (i.e. devoid of gonadotropin receptor-specific antibodies), enhanced the FSH-induced reporter gene activation by a factor 2. IgM obtained from the anti-FSHR sera inhibited the receptor activation as compared to control IgM (−60%), whereas IgM from the anti-LHR sera did not (Fig. 8).

3.4. Effect of gonadotropin receptor vaccines on body growth

No significant effect of vaccines on the body growth (specific growth rate) was observed in Experiments I and II (Fig. 9A and B). In Experiment III, both vaccine treatments inhibited the body growth rate of the females (as compared to adjuvant treated and untreated controls ($p < 0.01$) (Fig. 9C).

4. Discussion

We report here on the first trial to disrupt the gametogenesis process in rainbow trout via an active immunization against specific epitopes of the LH or the FSH receptors.

The phage display vaccination procedure we used was the only available strategy by which antagonists of gonadotropin receptors could be tested. In this study it proved to be efficient in obtaining an immune response coinciding with important disorders in male and female gonadal function.

Our data showed that vaccines against Fshr or Lhr or a combination of both, induced different inhibitory effects on the progress of gametogenesis. Due to these vaccine-specific inhibitory effects on different physiological processes, we can exclude an effect of the vector itself. As demonstrated in other species, injection of non-recombinant fd phages never induced any effect on reproductive parameters (Abdennebi et al., 1999). Furthermore, in a complementary experiment we verified that only the vaccination with phage displaying a gonadotropin receptor peptide had inhibitory effects on trout spermatogenesis, while phage displaying random peptides had no significant effect.

Recombinant phages expressed 17 amino acids of the trout Lhr or Fshr: although these two sequences share four residues, it is far from being enough to induce cross-reactive antibodies. In previous studies in mammals, we demonstrated that immunization with such hybrid bacteriophages induced immunity against the targeted gonadotropin receptor (Abdennebi et al., 1999). To take into account the fact that the response type observed in a mammal is not necessarily conclusive for the situation in trout, we purified IgM from immunized fish and tested their ability to inhibit the response to FSH of COS-7 cells expressing the Fshr. In the presence of control IgM preparation the FSH-induced receptor activation was strongly increased, possibly due to a non-specific serum effect. In these circumstances an inhibitory effect of anti-FSHR IgM on the FSH response could not be demonstrated when comparing the conditions "FSH alone" and "FSH plus anti-FSHR IgM". However, Fshr activation appeared significantly inhibited by the anti-FSHR IgM when compared to treatment with FSH plus control IgM. This effect appears specific since the anti-LHR IgM had no detectable effect. Although no definitive conclusion could be drawn from this experiment, the data strongly suggest that the biological effects of the vaccinations result from a specific action on the targeted gonadotropin receptor.

We found that immunization against Lhr or Fshr, alone or in combination, resulted in inhibition/delay of the spermatogenic and vitellogenic processes, and had inhibitory effects on plasma steroid levels. In fish, FSH is generally considered to regulate early phases of oogenesis and spermatogenesis, while LH is responsible for final oocyte maturation and milt production (Schulz and Miura, 2002; Ko et al., 2007). Due to potential cross reactivity of these hormones on fish receptors, and because FSH receptor expression was detected in both interstitial and intratubular compartment (Ohta et al., 2007; Garcia-Lopez et al., 2009), the delineation of the

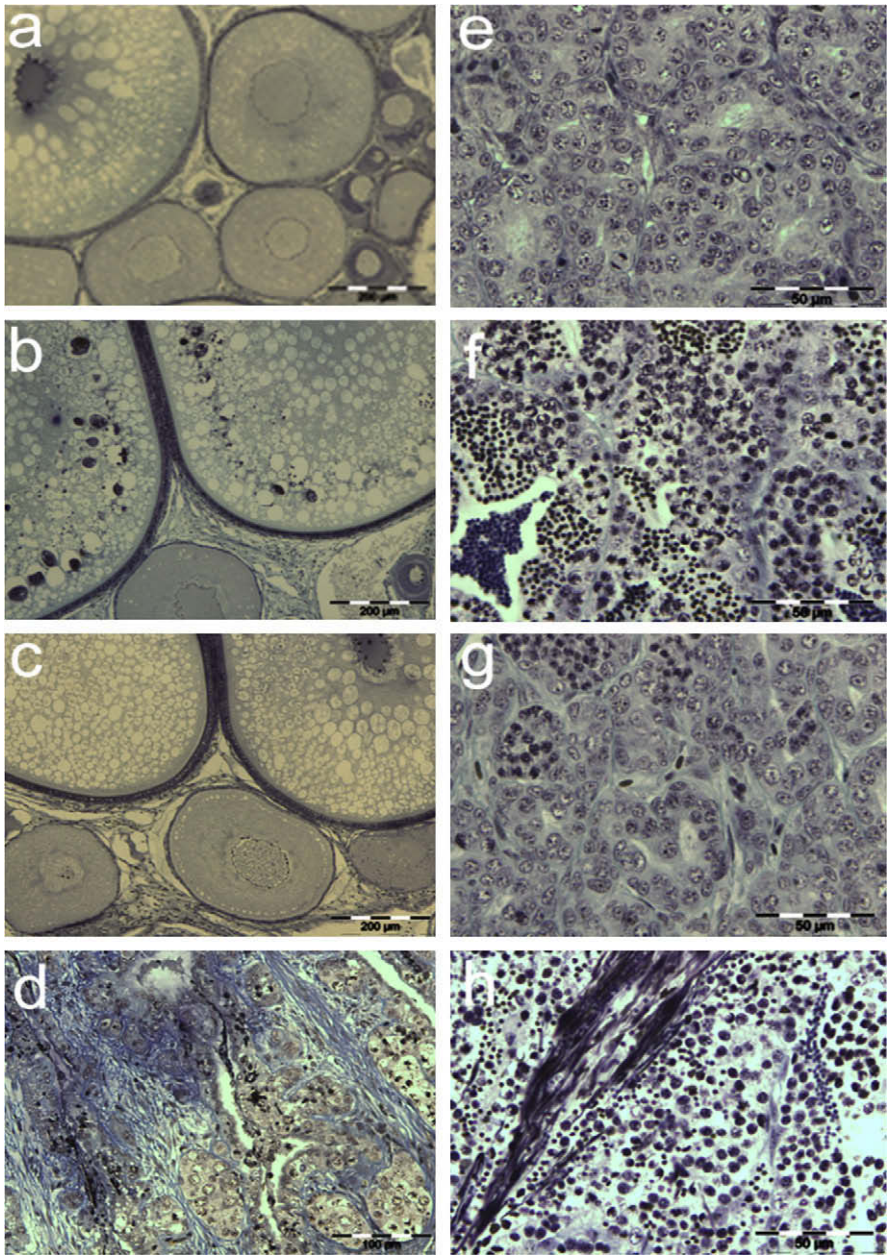

Fig. 7. Histological pictures of gonads from females in Experiment III (a–d, 10×) and males in Experiment II (e–h, 40×). Fixed gonads were dehydrated and embedded in paraffin, and 5 µm sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue. Gonads a and e: D0; b: D55, adjuvant treatment; c: D55 anti-LHR treatment; d: D49 anti-LHR treatment; f: D67 adjuvant treatment; g: D67 anti-FSHR + LHR; h: D67 anti-FSHR.

biological functions of the receptors remains unclear. Vaccination against the gonadotropin receptors at chosen crucial stages of puberty or during gametogenesis could therefore be an alternative approach to elucidate the physiological roles of each gonadotropin/receptor signaling pathway in fish reproduction.

Complementary studies will be necessary to demonstrate the precise mechanisms involved in the inhibitory effects described here. However, we can put forward some suggestions on physiological functions of Fshr in fish gametogenesis, for which little data is available.

We aimed at investigating the influence of the reproductive stage on the response to immunisation. Immunization procedure started either before (December for Experiment I and complementary experiment) or after (April for Experiment II) sexual maturation had started. In male, when immunization against Fshr peptides began early in the reproductive cycle, we observed an increased proportion of totally immature fish at the end of the experiment; this observation supports the idea that FSH/Fshr signaling is physiologically involved in the initiation of sexual maturation. This is consistent with our previous observation of a transient increase in FSH blood plasma levels in the early stage of trout maturation (Gomez et al., 1999) and with the demonstration of FSH receptor expression in trout testis at that stage (Miwa et al., 1994; Kusakabe et al., 2006; Sambroni et al., 2007). The early physiological role of Fshr could play a part in the initiation of spermatogonial proliferation, as suggested from observation on FSH action *in vitro* (Loir, 1999; Ohta et al., 2007). Fshr could also be involved in the regulation of Sertoli cell proliferation/maturation, which was found to occur primarily in pre-meiotic cysts (Schulz et al., 2005). Our data in trout are consistent with our previous observations in mammals

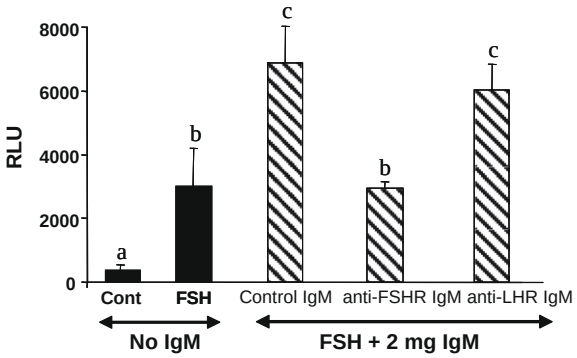

Fig. 8. FSH responsiveness of COS-7 cells expressing trout Fshr and effect of IgM preparations from sera of control or immunized fish. Cells were pre-incubated for 2 h with 2 mg/ml of IgM from control, anti-FSHR or anti-LHR immunized fish before adding FSH (500 ng/ml) for another 6 h. Hormone-induced cAMP production was indirectly quantified by measuring the reporter gene luciferase activity. Black bars: stimulation with FSH alone and hatched bars: stimulation with FSH in presence of IgM. Different letters denote significant differences among treatments ($p < 0.05$).

where Fshr vaccines delayed the onset of puberty and reduced fertility in male mice (Abdennebi et al., 2003).

We found that when immunization started during active spermatogenesis, only vaccination against both receptor peptides was potent enough to significantly inhibit further evolution through the late stages of meiosis and spermiogenesis. These data suggest that Lhr and Fshr play a part in post meiotic cyst maturation in trout, which is consistent with our previous observation of large increases in both plasma FSH and LH levels, correlated with the number of cysts which enter spermiogenesis (Gomez et al., 1999). This may differ from mice in which only LH and Lhr are essential for spermiogenesis (Kumar, 2005). In Experiment III sex ratio in favor of females and unexplained mortality rate in all groups drastically reduced the number of experimental males. However, a lower GSI and delayed spermatogenesis were also observed in the few males of the anti-FSHR and anti-LHR treated groups (data not shown).

In female trout already engaged in vitellogenesis, anti-FSHR or anti-LHR immunizations inhibited the evolution of the vitellogenesis process. Vaccination against Lhr peptides was more efficient than that against Fshr peptides in slowing down lipid globule and vitellus accumulation in oocytes. This is surprising because in female trout at the beginning of the reproductive cycle, FSH is the predominant gonadotropin, with peak concentrations occurring during early vitellogenesis (Breton et al., 1998). Also, FSH has been found to be specifically involved in vitellogenic development of oocytes in the rainbow trout (Tyler et al., 1991, 1997; Ko et al., 2007). This may reflect the fact that in fish we cannot easily assimilate the effect of one gonadotropin with the activity of its cognate receptor. However, LH is capable of steroidogenic actions in the vitellogenic ovary (Suzuki et al., 1988; Planas et al., 1997); Lhr transcripts are present at that stage and increase during the accumulation of lipid droplets (Campbell et al., 2006). Our observations indicate that Lhr function on early vitellogenesis should be considered.

Due to the small number of females in our experiment, it was not possible to assess whether the effects on the kinetics of vitellogenesis would also be reversible after immunization treatment stopped. However we found that 10 weeks after the last vaccine injection, the inhibitory effects of anti-FSHR + LHR immunization on spermatogenesis could no longer be detected, suggesting complete reversibility of the inhibitory effects of the vaccines, following an interruption of the treatment.

Vaccination against Lhr and Fshr had a global inhibitory effect on plasma testosterone and 11KT levels in male and on plasma testosterone and estradiol in female trout. This is in accordance with the

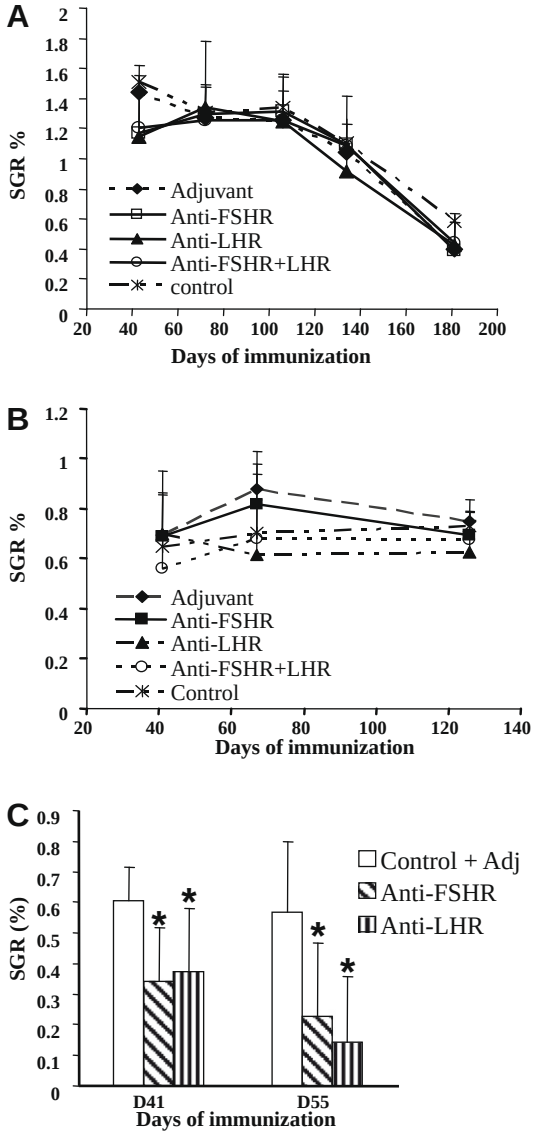

Fig. 9. Specific growth rate (SGR) calculated for the three experiments according to the following formula: $SGR = \frac{\mathrm{Ln}M_f - \mathrm{Ln}M_i}{t} \times 100$ where t represents the period in days, Ln the Neperian logarithm, M_i and M_f the initial and the final body weight, respectively. (A) Males in Experiment I, (B) males in Experiment II and (C) females in Experiment III.

fact that in fish, both gonadotropins play a prominent role in the regulation of steroidogenesis (Planas and Swanson, 1995). It also supports the hypothesis that FSH plays a central role in male puberty, possibly through the control of steroid production. Interestingly, the delay of sexual maturity previously observed after immunization against Fshr in two breeds of pre-pubertal goats was also associated with a low testosterone level for several months without alteration of the LH levels (Abdennebi et al., 2003). The inhibitory effects of vaccinations on testosterone and 11KT were more pronounced in the later stages of trout maturation (D180 in Experiment I), when gonad steroidogenesis is very active and highly regulated by pituitary hormones. Although testosterone plasma levels were affected by the three vaccines, not all immunizations had detectable effects on male gametogenetic development. Only anti-FSHR in Experiment I, and the combination anti-FSHR + LHR in Experiment II, significantly affected testicular maturation. We cannot exclude the possibility that in the other experimental conditions intragonadal steroid concentrations were still sufficient to support spermatogenesis. However, it most likely reveals the fact that factors

under FSH control, other than steroid hormones, are necessary to trigger normal gametogenesis.

In several teleost species, reduced growth and lower flesh quality coincides with sexual maturation, and precocious maturation is a problem in commercial fish farming. Therefore, reliable methods to control the onset of puberty are required. Different strategies have been developed in order to circumvent this drawback. For example, production of sterile triploid fish (Chourrout, 1980, 1984), combined with production of all-female populations (through hormonal phenotypical sex inversion of broodstock for species where females are less precocious than males; (Pandian and Sheela, 1995; Lee et al., 2004). These strategies are not necessarily applicable to all species, or could be found to be hazardous for the environment, and further techniques are needed for the control of maturation. One of our aims in studying the effect of immunization against gonadotropin receptors was to assess this approach as a possible method of controlling sexual maturity in trout.

Despite the use of adjuvant and multiple booster injections, the magnitude of immune response in the trout remained relatively weak when compared to that observed in mammals, as already commented on in previous studies. The temperature at which the fish are reared could explain these observations (Gudding et al., 1999). The somehow moderate effects of the anti-gonadotropin receptor vaccinations on trout reproductive function may be linked to this relatively weak immune response. The adjuvant used, the rhythm of the injections and the rearing temperature are important parameters that could certainly be optimized. Efficiency in blocking pubertal maturation might also be improved by starting immunization much earlier prior to puberty onset (Graf et al., 1997; Abdennebi et al., 1999). Finally, DNA vaccines might also be a technology of choice, in fact recently DNA vaccination against infectious hematopoietic necrosis virus was found to be effective in the trout (Kurath et al., 2006). In any case the procedure should not negatively affect fish health or body growth. The procedure used here affected growth in only one experiment out of three, which remains unexplained and could not be specifically related to the female sex of the experimental fish.

In conclusion, we show for the first time that vaccination against the gonadotropin receptors displayed marked antagonistic effects on gametogenesis in fish. The data suggest that these effects depend on the targeted receptor and differ according to the stage of development and/or to the endocrine status of the fish. Such a strategy could become an alternative approach to elucidate the respective physiological roles of each of the gonadotropin receptors in fish reproduction. Immunosterilizing technology by vaccination against the gonadotropin receptors might also be a promising method for controlling the onset of puberty in aquaculture species, provided the formulation of an appropriate highly immunogenic vaccine can be achieved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2009.05.012.

References

Abdennebi, L., Chun, E.Y., Jammes, H., Wei, D., Remy, J.J., 2003. Maintenance of sexual immaturity in male mice and bucks by immunization against N-terminal peptides of the follicle-stimulating hormone receptor. Biol. Reprod. 68, 323–327.

Abdennebi, L., Couture, L., Grebert, D., Pajot, E., Salesse, R., Remy, J.J., 1999. Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. J. Mol. Endocrinol. 22, 151–159.

Breton, B., Govoroun, M., Mikolajczyk, T., 1998. GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. Gen. Comp. Endocrinol. 111, 38–50.

Campbell, B., Dickey, J., Beckman, B., Young, G., Pierce, A., Fukada, H., Swanson, P., 2006. Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biol. Reprod. 75, 34–44.

Chourrout, D., 1980. Thermal induction of diploid gynogenesis and triploidy in the eggs of the rainbow trout (*Salmo gairdneri* Richardson). Reprod. Nutr. Dev. 20, 727–733.

Chourrout, D., 1984. Pressure-induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all-triploids, all-tetraploids, and heterozygous and homozygous diploid gynogenetics. Aquaculture 36, 111–126.

Fan, Q.R., Hendrickson, W.A., 2005. Structure of human follicle-stimulating hormone in complex with its receptor. Nature 433, 269–277.

Fostier, A., Billard, R., Breton, B., Legendre, M., Marlot, S., 1982. Plasma 11-oxotestosterone and gonadotropin during the beginning of spermiation in rainbow trout (*Salmo gairdneri* R.). Gen. Comp. Endocrinol. 46, 428–434.

Garcia-Lopez, A., Bogerd, J., Granneman, J.C.M., van Dijk, W., Trant, J.M., Taranger, G.L., Schulz, R.W., 2009. Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150, 357–365.

Gomez, J.M., Weil, C., Ollitrault, M., Le Bail, P.Y., Le Gac, F., 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 113, 413–428.

Graf, K.M., Dias, J.A., Griswold, M.D., 1997. Decreased spermatogenesis as the result of an induced autoimmune reaction directed against the gonadotropin receptors in male rats. J. Androl. 18, 174–185.

Gudding, R., Lillehaug, A., Evensen, O., 1999. Recent developments in fish vaccinology. Vet. Immunol. Immunopathol. 72, 203–212.

Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (fsh) and luteinizing hormone (lh): production of biologically active recombinant fsh and lh by Drosophila s2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938–946.

Ko, H., Park, W., Kim, D.J., Kobayashi, M., Sohn, Y.C., 2007. Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies. J. Mol. Endocrinol. 38, 99–111.

Kumar, R.S., Ijiri, S., Trant, J.M., 2001. Molecular biology of the channel catfish gonadotropin receptors: 2. Complementary DNA cloning, functional expression, and seasonal gene expression of the follicle-stimulating hormone receptor. Biol. Reprod. 65, 710–717.

Kumar, T.R., 2005. What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? Reproduction 130, 293–302.

Kurath, G., Garver, K.A., Corbeil, S., Elliott, D.G., Anderson, E.D., LaPatra, S.E., 2006. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 24, 345–354.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189, 541–554.

Lee, P., King, H., Pankhurst, N., 2004. Preliminary assessment of sex inversion of farmed Atlantic salmon by dietary and immersion androgen treatments. N. Am. J. Aquacult. 66, 1–7.

Loir, M., 1999. Spermatogonia of rainbow trout: II. In vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. Mol. Reprod. Dev. 53, 434–442.

Mateos, J., Mananos, E., Carrillo, M., Zanuy, S., 2002. Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 132, 75–86.

Miwa, S., Yan, L., Swanson, P., 1994. Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. Biol. Reprod. 50, 629–642.

Nagahama, Y., Miura, T., Kobayashi, T., 1994. The onset of spermatogenesis in fish. Ciba Found. Symp. 182, 255–267.

Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., Nagahama, Y., 1999. Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem. Biophys. Res. Commun. 263, 584–590.

Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. Biol. Reprod. 77, 970–977.

Pandian, T.J., Sheela, S.G., 1995. Hormonal induction of sex reversal in fish. Aquaculture 138, 1–22.

Planas, J.V., Goetz, F.W., Swanson, P., 1997. Stimulation of brook trout ovarian steroidogenesis by gonadotropins I and II is mediated by the cyclic adenosine 3′,5′-monophosphate/protein kinase A pathway. Biol. Reprod. 57, 647–654.

Planas, J.V., Swanson, P., 1995. Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. Biol. Reprod. 52, 697–704.

Planas, J.V., Athos, J., Goetz, F.W., Swanson, P., 2000. Regulation of ovarian steroidogenesis in vitro by follicle-stimulating hormone and luteinizing hormone during sexual maturation in salmonid fish. Biol. Reprod. 62, 1262–1269.

Planas, J.V., Swanson, P., Dickhoff, W.W., 1993. Regulation of testicular steroid production in vitro by gonadotropins (GTH I and GTH II) and cyclic AMP in coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 91, 8–24.

Rao, A.J., Ramachandra, S.G., Ramesh, V., Couture, L., Abdennebi, L., Salesse, R., Remy, J.J., 2004. Induction of infertility in adult male bonnet monkeys by immunization with phage-expressed peptides of the extracellular domain of FSH receptor. Reprod. Biomed. Online 8, 385–391.

Remy, J.J., Couture, L., Rabesona, H., Haertle, T., Salesse, R., 1996. Immunization against exon 1 decapeptides from the lutropin/choriogonadotropin receptor or the follitropin receptor as potential male contraceptive. J. Reprod. Immunol. 32, 37–54.

Sambroni, E., Le Gac, F., Breton, B., Lareyre, J.J., 2007. Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. J. Endocrinol. 195, 213–228.

Schulz, R., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43–56.

Schulz, R.W., Andriske, M., Lembke, P.J., Blum, V., 1992. Effect of salmon gonadotropic hormone on sex steroids in male rainbow trout: plasma levels and testicular secretion in vitro. J. Comp. Physiol. B 162, 224–230.

Schulz, R.W., Vischer, H.F., Cavaco, J.E., Santos, E.M., Tyler, C.R., Goos, H.J., Bogerd, J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 129, 407–417.

Schulz, R.W., Menting, S., Bogerd, J., Franca, L.R., Vilela, D.A.R., Godinho, H.P., 2005. Sertoli cell proliferation in the adult testis—evidence from two fish species belonging to different orders. Biol. Reprod. 73, 891–898.

So, W.K., Kwok, H.F., Ge, W., 2005. Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits—their spatial–temporal expression patterns and receptor specificity. Biol. Reprod. 72, 1382–1396.

Suzuki, K., Nagahama, Y., Kawauchi, H., 1988. Steroidogenic activities of two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71, 452–458.

Taillard Éric, D., Waelti, Philippe, Zuber, Jacques, 2008. Few statistical tests for proportions comparison. Eur. J. Oper. Res. 185, 1336–1350

Tilly, J.L., Aihara, T., Nishimori, K., Jia, X.C., Billig, H., Kowalski, K.I., Perlas, E.A., Hsueh, A.J., 1992. Expression of recombinant human follicle-stimulating hormone receptor: species-specific ligand binding, signal transduction, and identification of multiple ovarian messenger ribonucleic acid transcripts. Endocrinology 131, 799–806.

Tyler, C.R., Pottinger, T.G., Coward, K., Prat, F., Beresford, N., Maddix, S., 1997. Salmonid follicle-stimulating hormone (GtH I) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. Biol. Reprod. 57, 1238–1244.

Tyler, C.R., Sumpter, J.P., Kawauchi, H., Swanson, P., 1991. Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 84, 291–299.

Vischer, H.F., Granneman, J.C., Linskens, M.H., Schulz, R.W., Bogerd, J., 2003. Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. J. Mol. Endocrinol. 31, 133–140.

Weil, C., Bougoussa-Houadec, M., Gallais, C., Itoh, S., Sekine, S., Valotaire, Y., 1995. Preliminary evidence suggesting variations of GtH1 and GtH2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 100, 327–333.

Yan, L., Swanson, P., Dickhoff, W.W., 1992. A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biol. Reprod. 47, 418–427.

Active immunization against gonadotropin receptors can impair reproductive function in rainbow trout

by

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ABSTRACT. - The consequences of immunization against specific regions of gonadotropin receptors were analysed in rainbow trout, on gonadal development and plasma testosterone levels. Males immunized against LHR alone or in combination with FSHR, showed a decrease in mean GSI, related to an inhibition of the spermatogenetic process; females immunized against FSHR or LHR exhibited delayed vitellogenesis. Vaccination against FSHR, and to a less extent against LHR, had a global inhibitory effect on plasma testosterone levels.

Key words. - Rainbow trout - Gametogenesis - Immunization - FSH and LH receptors.

Introduction

Precocious sexual maturity is a drawback encountered in fish aquaculture and reliable methods to control the onset of puberty are required. In mammals, previous studies demonstrated that targeting specific regions of LH and FSH receptors, through the immune pathway, induced impairment of adult fertility and sexual maturity. We aimed to study the effect of immunization against specific regions of each receptor on sexual maturity in rainbow trout.

Method

Filamentous phages displaying specific decapeptides of the extracellular region of the FSH receptor (A: NTITHMPTHI and B: THIPKNTTDL) and of the LH receptor (C: NNITEKSVPT and D: VPTSERGPRL) were engineered (Abdennebi *et al*., 1999) and used as peptidic vaccines in rainbow trout. Male and female already engaged in their first reproductive cycle, received either adjuvant alone or one of the 3 following vaccines: anti-LHR (C+D), anti-FSHR (A+B), or a combination of both vaccines (anti-FSHR+LHR; males only), through 5 injections over an 8 weeks period. Antibody titration was determined by ELISA against phages. The consequences of immunization on gonadal development were analysed by measuring the gonad weight and by histological examination, 12 days and 10 weeks after the final injection. Serum testosterone (T) was determined by radioimmunoassay from blood collected at four different times during the experiment.

Results and discussion

Immune response: The humoral response against phages could be detected 20 days after the first booster injection, kept increasing up to one month after the second booster injection, then leveled off until the end of the immunization period.

Gametogenesis development (Tab. I): 12 days after the end of treatment, males immunized against LHR alone or in combination with FSHR, showed a decrease in mean GSI, as compared to control. In the anti-FSHR+LHR group, evolution of the spermatogenetic process appeared inhibited, as observed by histological analysis of the gonads: 30% of the males presented only spermatogonia (against 0% in adjuvant or anti-FSHR group), and only 40% of the males had reached the stage of initiation of spermiogenesis and first appearance of spermatozoa (against 90% in adjuvant or anti-FSHR group). Inhibitory effects of immunization were not longer detected 10 weeks after the final injection, showing the rapid and complete reversibility of the process.

As regarding females, 12 days after the end of treatment, vitellogenesis appeared inhibited: only 40% and 25% had reached the stage of accumulation of yolk globules (exogenous vitellogenesis) in the anti-FSHR and anti-LHR immunized groups, respectively, compared to 75% in adjuvant group.

Testosterone: In male as in female trout, vaccination against FSHR, and to a less extent against LHR, had a global inhibitory effect on plasma testosterone levels. That became

Immunization type	GSI		Gametogenesis development		Testosterone	
	Male	female	Male	female	Male	female
Anti-FSHR	=	=	=	↓	↓	↓
Anti-LHR	↓	=	↓	↓↓	↓↓	↓
Anti-FSHR+LHR	↓	Not tested	↓↓	Not tested	↓	Not tested

Table I. - Summary of the observed effects of immunization against gonadotropin receptors on reproductive function in rainbow trout.

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statistically significant after the fourth vaccine injection (day 49 of the experiment).

Conclusion

We show for the first time that the anti-receptor vaccination strategy could have specific antagonistic effects on gametogenesis and steroidogenesis in fish. This strategy could be a helpful approach to elucidate the respective physiological roles of each gonadotropin in fish reproduction, by blocking specifically their receptors at chosen crucial stages of puberty or along gametogenesis. Vaccination against the gonadotropin receptors might also be a promising method to control sexual maturation, although the immunization protocol has to be improved to be practicable in fish farming.

References

ABDENNEBI L., COUTURE L., GREBERT D., PAJOT E., SALESSE R. & J.J. REMY, 1999. - Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. *J. Mol. Endocrinol*., 22: 151-159.

Active immunization against gonadotropin receptors can impair reproduction function in rainbow

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Context - *Precocious sexual maturity is a drawback encountered in fish aquaculture and reliable methods to control the onset of puberty are required. In mammals, previous studies demonstrated that targeting specific regions of LH and FSH receptors, through the immune pathway, induced impairment in adult fertility and sexual maturity.*

Objective - to study the effect of immunization against specific regions of each receptor on gonadal functions in rainbow trout.

Method

Peptide vaccines preparation

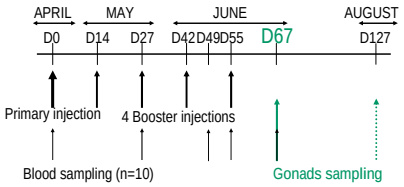
Insertion of specific oligonucleotides encoding rainbow trout FSH and LH receptors decapeptides in the protein p8 of phage Fd (*)

FSHR A: NTITHMPTHI FSHR B: THIPKNTTDL

LHR C: NNITEKSVPT LHR D: VPTSERGPRL

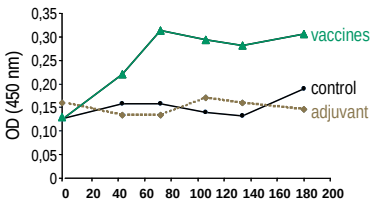
Type of immunization	Vaccines components
Adjuvant	0.25 ml of PBS + 0.25 ml of an experimental adjuvant GERBU«734»
Anti-FSHR	peptides A and B: 0.5 mg of each phage diluted in 0.25 ml of PBS + 0.25 ml of adjuvant
Anti-LHR	peptides C and D: 0.5 mg of each phage diluted in 0.25 ml of PBS + 0.25 ml of adjuvant
Anti-FSHR + LHR group	peptides A + B and peptides C + D, 0.25 mg of each construction (total 1 mg of phage in 0.5ml of vaccine)

Immunisation schedule (n=20 per group)

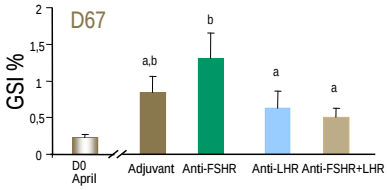


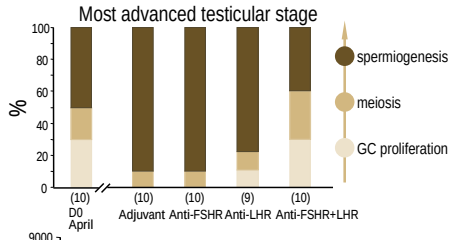
Results

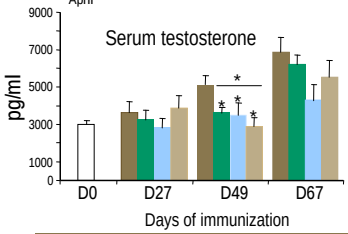
Humoral response against phages (ELISA)



Males

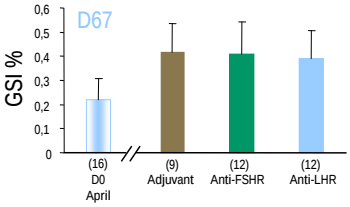


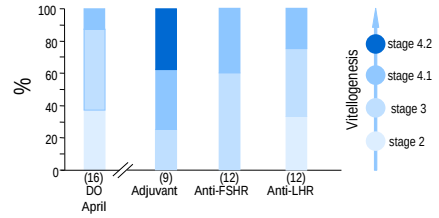


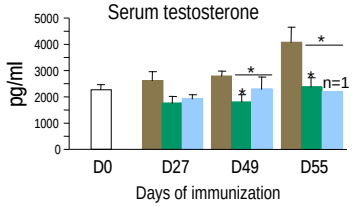


✓GSI decreased with anti-LHR
✓Inhibition of spermatogenesis
✓Inhibition of testosterone
✓Recovery on day 127

Females







✓GSI unchanged
✓Delayed vitellogenesis
✓Inhibition of testosterone

Conclusion

The phage display vaccination procedure tested here proved to be efficient to obtain an immune response related to important disorders in male and female gonadal function

Active immunization against the gonadotropin receptors could be an alternative approach to elucidate the respective physiological roles of each gonadotropin in fish reproduction

Active immunization against the gonadotropin receptors might also be a promising method to control the onset of puberty in aquaculture species.

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Résultats - partie 2

Comment les 2 gonadotropines régulent-elles l'expression génique dans le testicule ?

Fsh et Lh ont des effets communs et des effets distincts

Deux grands mécanismes d'action de la Fsh ont été mis en évidence

Des voies originales d'action de Fsh sur la gamétogenèse sont proposées

Chez les mammifères mâles, FSH régule les capacités spermatogénétiques de la gonade en agissant directement sur les cellules de Sertoli, tandis que LH induit la synthèse de stéroïdes par les cellules de Leydig. Ce schéma de la régulation des fonctions testiculaires par les 2 gonadotropines peut ne pas être pertinent chez les poissons. En effet, Fsh et Lh sont capables de stimuler efficacement la stéroïdogenèse et les récepteurs de la Fsh sont présents sur les cellules de Leydig chez plusieurs espèces de téléostéens. Ces particularités, entre autres, font que les rôles respectifs de Fsh et de Lh ne sont pas encore clairement établis. De plus, les gènes qui sous-tendent leur action sur les fonctions testiculaires ont été très peu étudiés chez les poissons. Nous avons donc cherché dans un premier temps à identifier des gènes régulés par Fsh et par Lh afin de comprendre si les 2 hormones exercent les mêmes effets génomiques au sein du testicule de truite. Puis dans un deuxième temps, nous nous sommes intéressés au mécanisme d'action de la Fsh sur les fonctions testiculaires en cherchant à savoir si les effets de la Fsh sont relayés exclusivement par la production de stéroïdes.

La démarche a consisté à réaliser des incubations *in vitro* d'explants issus de testicules à des stades précoces de maturation gonadique (stades I à III), pendant 96h, en présence ou en absence de Fsh et de Lh dans une première série et, dans une deuxième série, en présence de Fsh seule ou en combinaison avec un inhibiteur de la stéroïdogenèse, le trilostane. Enfin dans une troisième série, nous avons réalisé des incubations en présence de Fsh et d'androgènes (11KT et MT). Nous avons analysé les variations du transcriptome induites par les traitements hormonaux à l'aide de puces à ADNc pour les 2 premières séries d'incubation et par qPCR sur quelques gènes candidats pour la 3^{e} série.

En résumé, dans le premier manuscrit, nous montrons que Fsh et Lh ont des effets communs et distincts sur l'expression des gènes. Sur l'ensemble des gènes différentiellement exprimés, environ 1/3 sont régulés de façon similaire par Fsh et par Lh, tandis que 20% le sont préférentiellement par Fsh et 42 % par Lh. Une grande proportion des gènes est exprimée dans les cellules somatiques et présente un profil d'expression au cours du cycle de maturation gonadique cohérent avec les profils de sécrétion des gonadotropines. La fonction de la plupart des gènes préférentiellement régulés par Lh reste difficile à mettre en lien avec le développement spermatogénétique. Certains pourraient être impliqués dans le devenir des cellules testiculaires (*pvrl1*, *bty*) ou dans la maturation du sperme (*ehmt2*, *racgap1*) mais nécessitent de plus amples investigations.

Notre étude démontre, qu'en plus d'une action sur les gènes de la stéroïdogénèse, la Fsh coordonne l'expression de facteurs paracrines, stimulateurs ou inhibiteurs, connus pour

réguler la prolifération et la différenciation des cellules germinales. Certains de ces gènes appartiennent à des voies de régulation majeures, comme le système Igf (*igf1b* et *igfbp6*), la voie des Tgf béta (*amh*, *inha*, *inhba* et *fstl3*), la voie Wnt (*wisp1*). D'autres comme le gène *mdka*, codant pour une midkine, devront faire l'objet d'analyses complémentaires afin de déterminer leur rôle dans la spermatogenèse.

Dans le second manuscrit, nous mettons en évidence 2 mécanismes d'action de la Fsh. Le trilostane réduit significativement, voire abolit, la réponse à la Fsh de nombreux gènes (comme *wisp1*, *gapdhs*, *cldn11*, *inha*, *vt1* ou *dmrt1*) montrant que chez les poissons, une part importante de l'action de la Fsh suit des voies indirectes et nécessite la production de stéroïdes de la voie delta 4. Cette observation est confortée par le fait que la plupart des gènes régulés par Fsh via les stéroïdes sont aussi régulés par Lh.

En revanche, la réponse à Fsh de plusieurs autres gènes n'est pas modifiée en présence de trilostane. Une majorité de ces gènes sont préférentiellement régulés par Fsh, en comparaison avec Lh, ce qui suggère que les effets régulateurs spécifiques de Fsh sont indépendants de la production de stéroïdes. Ces effets concernent, entre autres, les gènes codant pour l'hormone anti-Müllerienne, la midkine A, l'angiopoietine-related protein7, les cyclines E1 et G1, l'hepatocyte growth factor activator et l'insuline-like growth factor 1b. Enfin, des effets antagonistes entre Fsh et les androgènes ont été trouvés, en particulier pour les gènes codant pour des facteurs clés de la stéroïdogénèse (*star*, *hsd3b1*, *cyp11b2-2*) ou de la voie Igf (*igf1b*).

Ces résultats ont été publiés dans 2 articles intitulés "Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis" et "Fsh controls gene expression in fish both independently of and through steroid mediation".

Ils ont également été présentés sous forme de communications orales au 9^{e} Symposium International de Physiologie de la Reproduction des Poissons à Cochin en Inde (9th ISRPF, 9-14 août 2011) (Sambroni *et al.* 2011) et à la conférence Européenne des Endocrinologistes à Zürich en Suisse (CECE 2012, 21-25 août 2012) et sous forme d'un poster au 7^{e} Symposium International d'Endocrinologie des Poissons à Buenos Aires en Argentine (7th ISFE, 3-6 septembre 2012).

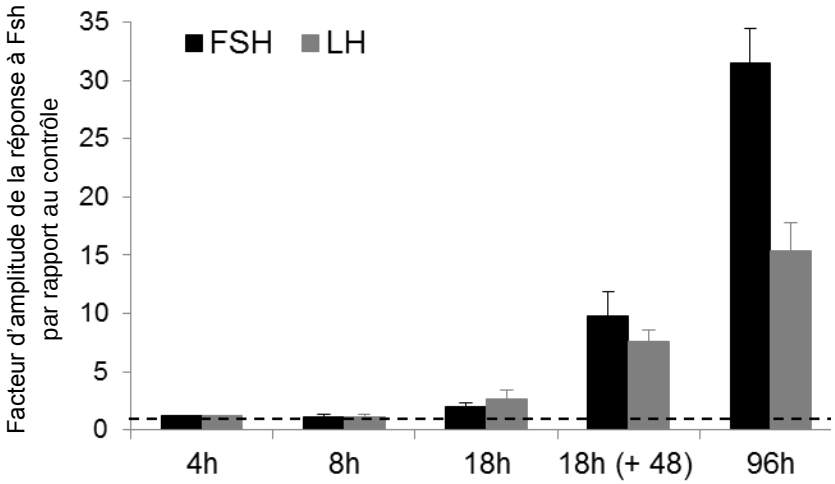

Figure 9 : Expression de *star* mesurée par qPCR dans du tissu testiculaire après incubations de durées variant de 4h à 4 jours en présence de Fsh et Lh (Fold change).

Des expériences préliminaires ont été nécessaires avant d'établir les conditions optimales concernant la durée et les doses d'exposition aux hormones et de sélectionner les lots de membranes corrects, répondant à 2 critères de qualité importants : bonne amplification des ADNc et quantité déposée suffisante.

a. Choix des stades

Un objectif premier fort de l'équipe étant de préciser l'action de Fsh dans le déclenchement de la spermatogenèse à la puberté, notre étude s'est placée au moment de l'initiation de la spermatogenèse, chez l'animal pré pubère (stades I à IIIa). Cette phase du cycle est caractérisée par une prolifération active lente des spermatogonies A, les premières proliférations rapides des spermatogonies B au sein de cystes synchrones et, dans une très faible proportion de cystes, les premières apparitions de la méiose.

b. Choix des temps

Nous avons testé des temps d'incubation variant de 4 h à 4 jours. Les durées courtes d'incubation ne nous ont pas permis de voir une variation significative de l'expression des gènes. Ainsi, l'analyse statistique des données de 44 puces obtenues avec les ARNm extraits d'échantillons incubés à des temps courts (8h et 18h) n'a révélé que quelques transcrits différentiellement exprimés entre contrôle et traités, avec un risque faible de faux positifs. L'exemple du gène candidat *star*, illustré dans la figure 9, montre que son expression reste au niveau basal après 4 et 8 h d'incubation et qu'elle augmente progressivement avec la durée d'incubation pour atteindre un fort différentiel avec le niveau basal au bout de 96 h d'incubation.

a. Choix des hormones

Quand l'essentiel des expériences a été réalisé, nous disposions au laboratoire d'une quantité limitée de Fsh de truite purifiée mais plus de Lh purifiée. Nous n'avions pas non plus, à l'époque, accès aux hormones recombinantes. Nous avons donc utilisé la Lh de saumon chinook dont la séquence protéique est très homologue de celle de la Lh de truite. De plus, nous avions vérifié, dans notre étude de fonctionnalité des récepteurs (Sambroni *et al.* 2007), que le récepteur Lhcgr de truite répondait de la même façon à la Lh de truite et à la Lh de saumon chinook [EC_{50} similaire (~ 100ng/ml) et induction maximale de même ordre (x 10)].

Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis

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Abstract

The general rules established from mammalian species for the regulation of spermatogenesis by gonadotropins may not be fully relevant in fish. Particularly, Fsh is as potent as Lh to stimulate steroidogenesis and the Fsh receptor is expressed in Leydig cells. In seasonal breeders, Fsh is likely the major gonadotropin involved in spermatogenesis onset and Lh is required to support spermatogenesis progression and gamete release. However, the genes that relay the action of Fsh and Lh have been poorly investigated in fish. The present study was aimed at identifying gonadotropin-dependent genes expressed in the testis during fish puberty. We cultured pubertal trout testicular explants for 96 h, with or without gonadotropin, and analyzed transcriptome variations using microarrays. Fsh and Lh had similar effects on a large group of genes while other genes were preferentially regulated by one or the other gonadotropin. We showed that most of the responsive genes were expressed in somatic cells and exhibited relevant patterns during the seasonal reproductive cycle. Some genes preferentially modulated by Lh could be involved in testicular cell fate (*pvrl1* and *bty*) or sperm maturation (*ehmt2* and *racgap1*) and will deserve further examination. Besides Fsh's effects on the steroidogenic pathway, our study demonstrates that Fsh coordinates relevant stimulatory and inhibitory paracrine factors known to regulate early germ cell proliferation and differentiation. Some of these genes belong to major regulatory pathways including the Igf pathway (*igf1b*/*igf3* and *igfbp6*), the Tgfb pathway (*amh*, *inha*, *inhba*, and *fstl3*), the Wnt pathway (*wisp1*), and pleiotrophin (*mdka*).

Key Words
- Fsh
- spermatogenesis
- oncorhynchus mykiss
- transcriptome

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Introduction

In vertebrates, gametogenesis is mainly controlled by the brain–pituitary–gonad (BPG) axis and Fsh and Lh are the most important mediators regulating gonadal functions. Fsh and Lh biological actions depend on their binding to membrane receptors that belong to the G protein-coupled receptor superfamily (Levavi-Sivan *et al*. 2010). In mammals, each receptor is exclusively activated by its cognate ligand and is exclusively expressed in only one type of testicular somatic cells, Sertoli cells for FSHR and Leydig cells for LHCGR. In the last decade, the generation of mice lacking gonadotropin or gonadotropin receptors provided unique insight into the roles of gonadotropin signaling in the development and function of the male reproductive axis. In mice lacking FSH or FSH receptor (FORKO), the number of both Sertoli cells and germ cells is reduced, but animals remain fertile. Sperm quality defaults have also

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been observed in these mice (Abel *et al*. 2000, 2009, Kumar 2005, Huhtaniemi 2006). By contrast, mice lacking LH or LH receptor (LuRKO) are sterile due to an arrest of spermatogenesis beyond the round spermatid stage (Lei *et al*. 2001, Zhang *et al*. 2001, Kumar 2005). From these models, it is clear that FSH promotes the proliferation of Sertoli cells, increases the number of spermatogonia and enhances the entry of these cells into meiosis. On the other hand, LH is required for Leydig cell proliferation and maturation and is essential for the production of androgens that in turn allow the completion of meiosis and spermiogenesis.

In teleosts, the general scheme of regulation through the BPG axis is conserved but noticeable peculiarities exist regarding the effects of Fsh and Lh on the two main testicular functions, i.e. steroidogenesis and spermatogenesis. Fsh is capable, as well as Lh, of stimulating efficiently steroidogenesis in both immature and maturing testes (Planas & Swanson 1995). With regard to the gonadotropin receptors, the localization and binding or activation properties differ from what is generally admitted in mammals. The presence of Fshr has been described in the tubular compartment but also on Leydig cells in four species so far, i.e. eel, African catfish, zebrafish, and honeycomb grouper (Lei *et al*. 2001, Ohta *et al*. 2007, Garcia-Lopez *et al*. 2009, 2010, Alam *et al*. 2010). These studies have suggested that the steroidogenic potency of Fsh may result from the direct action of Fsh on Leydig cells. Not only does receptor localization differ, but cross-reactivity has also been described in binding assays with purified fish gonadotropins (Yan *et al*. 1992) and in functional studies using mammalian cell lines expressing fish receptors separately. In African catfish (Bogerd *et al*. 2001, Lei *et al*. 2001, Vischer & Bogerd 2003, Vischer *et al*. 2003), zebrafish (So *et al*. 2005), and Atlantic salmon (Andersson *et al*. 2009), a promiscuous activation of Fsh receptors has been reported using high Lh concentrations. However, in two salmonid species, amago salmon and rainbow trout, only Fsh was able to activate Fshr (Oba *et al*. 1999, Lei *et al*. 2001, Sambroni *et al*. 2007). The physiological relevance of the promiscuity of the piscine gonadotropin receptors therefore remains a matter of debate.

Many teleostean fish species are seasonal breeders and constitute original model species to understand the respective roles of Lh and Fsh in all vertebrates. In the highly cyclic salmonid species, gonadotropins are differentially secreted in the plasma through the reproductive cycle. In rainbow trout, Fsh is the only detectable circulating hormone during the first steps of gametogenetic development (Prat *et al*. 1996, Gomez *et al*. 1999). This suggests that Fsh may play an important role in the early cellular and molecular events that occur during spermatogenesis onset. Fsh has been suggested to be involved in Sertoli cell proliferation (Schulz *et al*. 2005) and was proposed to take an essential part in the initiation of spermatogenetic maturation through the production of androgens in eel (Ohta *et al*. 2007). In addition, we previously showed that purified Fsh was capable of stimulating spermatogonia proliferation in a co-culture system with spermatogonia and Sertoli cells (Loir 1999*b*).

The deciphering of FSH molecular targets was previously initiated using a transcriptomic approach in rat primary Sertoli cell culture (McLean *et al*. 2002) or in hypogonadal mice (Sadate-Ngatchou *et al*. 2004, Abel *et al*. 2009). FSH acted directly or indirectly to modulate gene expression in Sertoli and Leydig cells but affected only a few genes expressed in germ cells. To our knowledge, no large-scale analysis of the effect of LH on testicular gene expression has been reported in vertebrates.

The present study was aimed at identifying Fsh- and Lh-dependent genes expressed in trout testis using a transcriptomic approach to understand their respective effects during fish puberty. We demonstrated for the first time that Fsh and Lh have both common and distinct effects on gene expression. Our data indicate that Fsh coordinates different stimulatory and inhibitory pathways that are physiologically relevant for early germ cell development or for sperm maturation/excretion in fish. The literature survey shows that some of these pathways are conserved in vertebrates.

Materials and methods

Animals and *in vitro* organotypic culture

All-male population rainbow trout (*Oncorhynchus mykiss*) were obtained from the INRA experimental fish farm (PEIMA, Drennec, France) and kept in the laboratory facilities at 12 °C under a natural photoperiod. Fish were anesthetized in 0.5% 2-phenoxyethanol and killed by a blow to the head. Testes were removed, weighed, and kept on ice in synthetic L15 media modified by Loir (1999*a*) until preparation for culture.

According to the macroscopic aspect of the testes and to the calculated gonadosomatic index (GSI), two pools of gonads have been used: one pool obtained from 28 fish with a GSI $<0.1\%$ (mean GSI$=0.08\%$) and another one obtained from three fish with a GSI of between 0.2 and

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0.6% (mean GSI=0.38%). Testes were chopped into 4 mm^3 pieces, then pooled, and mixed. Some testis fragments were directly frozen in 1.2 ml of TRIzol reagent (Invitrogen) at −80 °C (T0 sampling) or fixed in Bouin's fluid to determine the testicular developmental stage, whereas the rest of the fragments were randomly distributed (60–80 mg/well) on Nunc polycarbonate membrane inserts in 24-well plates filled with 300 µl of modified L15 culture medium plus 2% Ultroser SF (Loir 1999*a*). Incubation was performed in six replicates in the absence or presence of purified salmonid Fsh or Lh (500 ng/ml for 96 h, at 12 °C). The medium was renewed after 48 h of incubation. At the end of incubation, tissues and culture media were centrifuged for 10 min at 200 ***g***. Tissues were frozen at −80 °C in 2 ml of TRIzol until RNA extraction. Culture media were frozen at −20 °C until steroid RIA.

Tissues fixed at T0 were dehydrated and embedded in paraffin, and 5 µm sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue. The maturity stages of the gonads were evaluated based on the presence and on the relative abundance (RA) of the different germ cell types, according to a classification described by Gomez *et al*. (1999). The pool of gonads obtained from fish with a GSI <0.1% contained pre-spermatogenetic testes in stage I (only A spermatogonia being present) and testes at stage II corresponding to the first appearance of B spermatogonia and active spermatogonia proliferation. The pool of gonads obtained from fish with a GSI >0.2% mainly constituted of testes in stage III corresponding to meiosis onset with the first appearance of spermatocytes and round spermatids.

Steroid measurement

To denature steroid-binding proteins, media were heated at 60 °C for 20 min and centrifuged at 3000 ***g***, at 4 °C for 15 min. Levels of 11-ketotestosterone (11KT) were measured by specific RIA in culture media according to Fostier *et al*. (1982). Each sample was assayed in duplicates. Assay sensitivity was 80 pg/ml and cross-reactivity with testosterone or adrenosterone was 10% and null with androstenedione.

cDNA microarray experiments

RNA extraction and cDNA target synthesis ▸ Total RNA was extracted using TRIzol reagent and further purified with the NucleoSpin RNA II kit (Macherey Nagel EURL, Hoerdt, France). RNA concentrations were quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Courtaboeuf, France) and RNA quality was determined using the Bioanalyzer 2100 (Agilent Technologies, Massy, France). For cDNA target labeling, 5 µg total RNA were reverse-transcribed for 2 h at 42 °C in the presence of radiolabeled dNTP (30 µCi [α-^{33}P]dCTP, 120 µM dCTP, 20 mM each dATP, dTTP, and dGTP) using an oligo(dT) primer and 400 units Superscript II reverse transcriptase (Invitrogen). RNA were degraded at 68 °C for 30 min with 1 µl of 10% SDS, 1 µl of 0.5 M EDTA, and 3 µl of 3 M NaOH. The reaction was then equilibrated at room temperature for 15 min and neutralized (10 µl of 1 M Tris–HCI and 3 µl of 2 M HCl).

Hybridization of microarrays and raw data production ▸ cDNA nylon membrane microarrays were generated by CRB GADIE (http://crb-gadie.inra.fr/) as described previously (Rescan *et al*. 2007). Prehybridization of the membranes was performed at 65 °C for 4 h in 5× Denhardt's, 5× SSC, and 0.5% SDS buffer. Labeled cDNA targets were denatured at 95 °C for 5 min and incubated with the arrays for 48 h at 65 °C in the same buffer. Membrane arrays were then washed three times for 1 h at 68 °C in the washing solution (0.1× SSC, 0.2% SDS) prior to a 48 h exposure to phosphor imaging plates. The plates were scanned using a FUJI BAS 500 and hybridization signal (Si) acquisition was done with BZscan software (Lopez *et al*. 2004). Each membrane was also hybridized with a ^{33}P-labeled oligonucleotide (5′-TAATAC-GACTCACTATAGGG-3′) that recognizes the vector part of every PCR product to quantify the amount of spotted cDNA (signal vector Vi).

Normalization procedure ▸ Expression data were normalized as described previously (Rolland *et al*. 2009). Briefly, raw data (Si) were corrected for the vector signal (Vi) proportional to the amount of spotted cDNA (Si/Vi). To avoid the bias affecting relative gene expression levels, the corrected signal of each spot was further multiplied by the median vector signal of all arrays for this same spot ((Si/Vi)×medVi). Expression values were then log2-transformed and subjected to a quantile–quantile normalization using AMEN software (http://sourceforge.net/projects/amen/; Chalmel & Primig (2008)). Raw data as well as a normalized expression file are available at the Gene-Omnibus public data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39465).

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Statistical and cluster analyses ▸ Non-informative clones for which too small an amount of cDNA was spotted (oligonucleotide signal less than three times the background level in more than 20% of samples) were removed from the analysis. Gonadotropin-responsive genes were then identified by comparing the control group with each of the treated groups (six replicates per group) at each stage (I–II and III), using the multi-class Limma statistical test with a false discovery rate (FDR) of 5% (Smyth *et al.* 2005). All differentially expressed transcripts, obtained by the union of the two lists of genes, were then subjected to a hierarchical classification (uncentered Pearson's correlation measure) to define groups of correlated gene expression (clusters). Trout cDNAs spotted onto the nylon membrane arrays were annotated based on the EST sequences and the search for orthologs in the sequenced and annotated genomes of four fish species, as described previously (Rolland *et al.* 2009) (column D in Supplementary information file 1, see section on supplementary data given at the end of this article), completed by the search for the best protein homologs based on the contig of EST provided by SIGENAE -som8 version (column G in Supplementary information file 1).

Meta-analysis ▸ Expression data of six testicular developmental stages and of isolated germ cell fractions (Rolland *et al.* 2009) were used to investigate the developmental profile and the cellular origin of gonadotropin-responsive genes. Samples in this dataset included immature testes in early stages containing only slowly dividing type A spermatogonia (stage I) or growing numbers of actively dividing type B spermatogonia (stages IIa and IIb), maturing testes also containing large numbers of meiotic spermatocytes (stage IIIb) and post-meiotic spermatids (stage V), spawning testes containing essentially mature spermatozoa (stage VIII), and fractions of isolated germ cells enriched in spermatogonia, spermatocytes, or spermatids. Differentially expressed genes identified in this previous study (F's statistic permutation test with a 5‰ FDR) were compared with gonadotropin-responsive genes.

Real-time quantitative PCR experiments

The quantitative PCR (qPCR) technique was used to confirm changes in expression for selected transcripts identified from the microarray analysis or to examine other transcripts of potential interest. Two micrograms of total RNA were subjected to RT using random hexamer primers and 200 units of MMLV reverse transcriptase (Promega) for 75 min at 37 °C in a final of volume of 25 μl. Real-time qPCR assays were performed on the StepOne Real-Time PCR System (Applied Biosystems, Villebon sur Yvette, France) using 4 μl of 1:30 diluted RT products, 1 μl of mixed oligo primers (0.6 μM for both reverse and forward primers), and 5 μl of Fast SYBR Green Master Mix (Applied Biosystems). The amplification program consisted of an initial denaturation step at 95 °C for 20 s, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. A final progressive increase in temperature (0.5 °C/s) has been carried out from 65 to 90 °C at the end of the amplification for melting curve analysis.

The RA was determined from a standard curve generated by performing serial dilutions of pooled RT products. Relative expression levels were normalized to the reference gene, *rps15* ($RA_{candidate}/RA_{rps15}$). The reference gene was chosen on the basis of its invariant expression over the spermatogenic testicular development (Rolland *et al.* 2009) and hormonal treatments. Its expression level also enabled its measurement at the same RT template dilution as selected candidate genes. All gene expression levels were measured in duplicates and statistical analyses were then performed using Statistica software using the non-parametric ANOVA of Kruskal–Wallis followed by the Mann–Whitney *U* test when a statistical difference ($P<0.05$) was observed between the groups in ANOVA.

Real-time PCR oligonucleotide primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and were verified with the oligoanalyzer 3.1 Web interface (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) to avoid self- and hetero-dimer formation as well as hairpin structures. They were also systematically matched (BLAST algorithm) against the SIGENAE trout contig collection (som.10 version) to avoid non-specific annealing to other transcripts. Efficiency of PCR amplification was determined using serial dilutions of pooled RT products and was closed to 100%. All primer sequences are provided in Supplementary Table 1, see section on supplementary data given at the end of this article.

Results

We investigated the effects of gonadotropins on the trout testicular transcriptome using testis explants at two stages of early gonadal maturation to focus on the initiation of spermatogenesis. Stages I–II are characterized by the presence of A spermatogonia and a small proportion of B spermatogonia. In stage III testis, B spermatogonia actively proliferates and meiosis progresses with the production of numerous spermatocytes and the appearance of round spermatids (Fig. 1).

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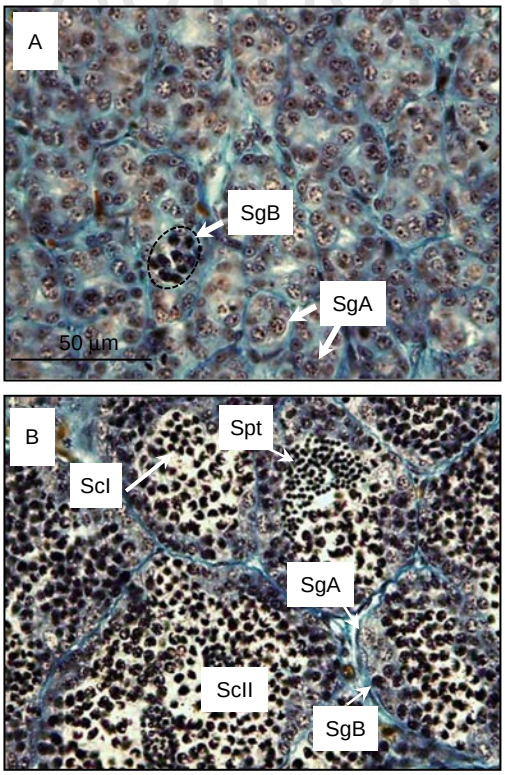


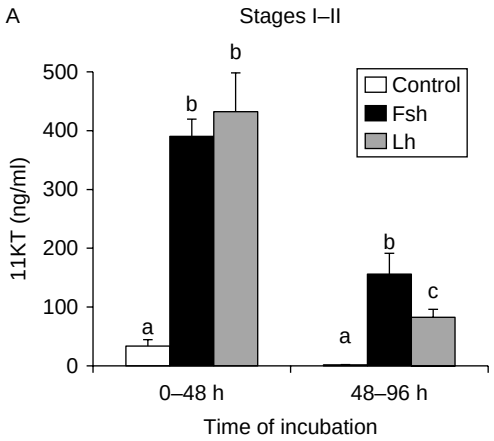
Figure 1
Histological analysis. Representative histology of the pooled testis explants used in the microarray experiment. (A) Stages I–II and (B) stage III. Tissues were fixed in Bouin's solution, dehydrated, and embedded in paraffin. Five micrometer sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue. SgA, A spermatogonia; SgB, B spermatogonia; ScI, primary spermatocytes; ScII, secondary spermatocytes; Spt, spermatids. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-12-0197.

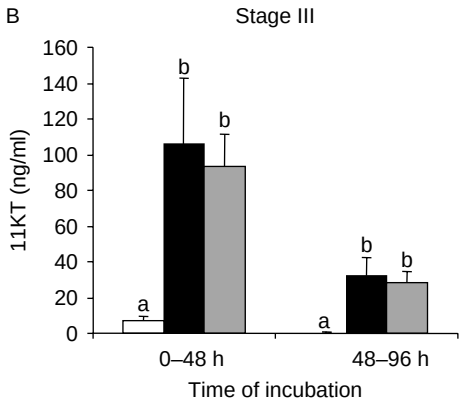
Both Fsh and Lh stimulated 11KT production over the culture period

To demonstrate the functional activities of the gonadotropins over the culture period, we first analyzed their effects on steroidogenesis at both studied stages. At stages I–II, 11KT accumulation in the culture media was highly increased (more than tenfold) when samples were stimulated in the presence of Fsh and Lh for 48 h (Fig. 2A). Although the basal 11KT production was altered after 48 h, gonadotropins still strongly stimulated 11KT production (about 90-fold) during the next 48 h period (Fig. 2A and C). The gonadotropin-induced steroid accumulation at 96 h was higher in the presence of Fsh compared with Lh. At stage III, the amplitude of the gonadotropin response was similar to that observed at stages I–II (Fig. 2B and C). Altogether, our data confirmed that Fsh and Lh efficiently stimulated steroid production in fish and indicated that the gonadotropin responsiveness of the cultured testes was maintained over the culture period.

Fsh and Lh had common and distinct effects on gene expression in rainbow trout testis

In preliminary experiments, short incubation times (4 and 8 h) did not allow the detection of the





C

		Stages I–II	Stage III
48 h	Fsh	11.6	15.3
	Lh	12.9	13.4
96 h	Fsh	90.2	95.8
	Lh	47.6	92.0

Figure 2
11KT production in culture media after 0–48 and 48–96 h of incubation in the absence or presence of purified salmonid Fsh or Lh at 500 ng/ml. (A) Testicular tissue explants at stages I–II; (B) testicular tissue explants at stage III. Culture media were replaced after 48 h. Each bar represents the mean ± s.d. of six replicates. Different letters indicate that the treatments are significantly different as determined by the non-parametric Mann–Whitney *U* test ($P<0.01$). (C) Fold changes compared with the control.

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gonadotropin-regulated genes. The number of genes and the amplitude of the response increased with the time of exposure (18–96 h incubation). In addition, we analyzed the effect of two doses of Fsh on a subset of the responsive genes (Supplementary Figure 1, see section on supplementary data given at the end of this article). For several of them, the dose of 100 ng/ml appeared suboptimal when compared with the dose of 500 ng/ml. Therefore, we incubated the testicular explants with 500 ng/ml gonadotropins for 96 h.

Microarray experiments provided data for 8175 well-measured clones. Unsupervised hierarchical classification of the stages I–II samples perfectly distinguished between the control and the different gonadotropin-treated samples (data not shown).

A Limma multi-class statistical analysis revealed that the relative abundance of 390 clones, corresponding to 372 non-redundant transcripts (NR), was significantly modulated following gonadotropin exposure (FDR <5%). In stage III, only 68 clones (65 NR) were found to be statistically differentially expressed and 55 clones (52 NR) were common with stages I–II. For a detailed analysis, we considered the 403 clones (385 NR) statistically differentially expressed in one or the other stage. Their complete list cannot be presented herein but is provided in Supplementary information file 1, together with the corresponding gene annotation, gene ontology (GO)-associated terms, and cluster information. The hierarchical classification carried out with the dataset obtained from the stages I–II samples separated six main clusters of genes with correlated variations (Fig. 3). Overall, an equal number of genes were upregulated or downregulated after the gonadotropin treatments. Interestingly, Fsh and Lh had similar effects for one-third of the regulated transcripts (cluster 2 'Up Fsh, Lh' and cluster 5 'Down Fsh, Lh' in Supplementary information file 1).

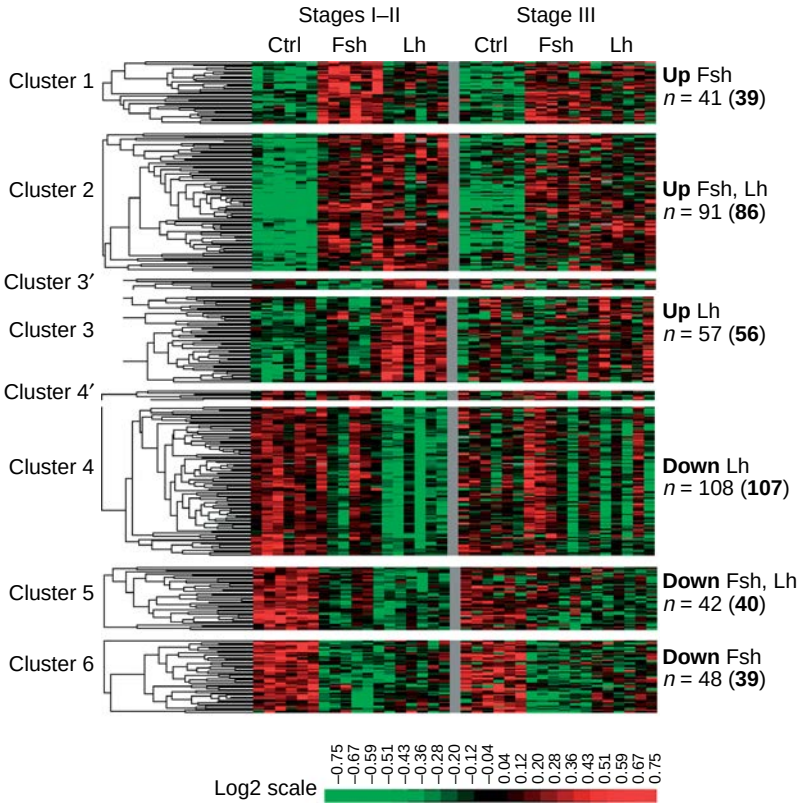

Figure 3

Hierarchical classification of the 403 clones regulated *in vitro* in the absence (Ctrl) or presence of Fsh or Lh at 500 ng/ml; 399 genes segregated into six main groups, corresponding to the genes up- or downregulated by both Lh and Fsh (Fsh, Lh) or preferentially regulated by one gonadotropin. Each line represents a clone and each column is a sample. Only the stages I–II genes were clustered, whereas the data in stage III are displayed on the same line. *n* is the number of clones in each cluster. The number of non-redundant genes is in bold characters.

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Table 1 Selected genes preferentially regulated by Lh with the highest differential between the Lh and Fsh responses. These genes from clusters 3 and 4 were identified as differentially regulated by Fsh and Lh in pairwise comparisons (Limma statistical test, FDR <5%)

Clone name	SwissProt/GenBank accession number	Gene symbol	Annotation/description	Predicted cellular origin
Cluster 3: 23 genes upregulated by Lh				
1RT27H07_B_D04	Q4SL44	*anxa11*	Annexin A11	–
tcbk0006.h.24	Q7SYB4	*pgam1b*	Phosphoglycerate mutase 1	–
tcad0003.j.02	P82861	*fdxr*	NADPH:adrenodoxin oxidoreductase, mitochondrial	–
tcay0006.k.10	Q6DRK0	*atp6v1f*	ATPase, H+ transporting, V1 subunit F	Germline
tcay0034.c.17	Q4T6N8	*ggtl3*	Gamma-glutamyltransferase 4 precursor	–
tcbk0003.a.18	Q4RT27	*mobkl1b*	Mps one binder kinase activator-like 1B	Germline
tcba0017.d.24	Q5U3H7	*ppm1db*	Protein phosphatase 1D	–
tcad0003.h.21	Q9DFH0	–	G-protein B1 subunit	Somatic
tcay0037.j.03	–	*atp2a3*	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Somatic
tcba0007.l.12	Q8AVH9	*ppp2ca*	Serine/threonine protein phosphatase	Gonia A
1RT60M18_C_G09	Q1L035	*cox3*	Cytochrome *c* oxidase subunit 3	Somatic
1RT92G03_A_D02	–	*fhl1*	Four and a half LIM domain protein 1	–
tcad0003.i.23	Q5DSV5	*bty*	Tripartite motif protein 'bloodthirsty-like'	–
tcay0034.d.21	Q6TNT2	*ankrd46*	Ankyrin repeat domain-containing protein 46	–
tcbk0028.h.02	–	*polr3a*	DNA-directed RNA polymerase III subunit RPC1	Somatic
tcay0009.o.12	–	*copa*	Coatomer subunit α (α-coat protein)	Gonia A
tcay0031.f.09	–	*sfrp2*	Secreted frizzled-related protein 2 precursor	Somatic
1RT129O10_C_H05	–	*serinc5*	Developmentally regulated protein TPO1	Germline
tcbk0052.i.13	B2RCV9	*slc22a13*	Highly similar to *Homo sapiens* solute carrier family 22, member 13	Somatic
1RT63G13_A_D07	–	*smyd1*	SET and MYND domain-containing protein 1	–
tcbk0036.m.23	–	*pvrl1*	Poliovirus receptor-related protein 1 precursor	–
tcbk0006.c.09	Q7ZVX9	*txndc9*	Thioredoxin domain containing 9	Germline
tcba0001.c.18	–	*cops5*	COP9 signalosome complex subunit 5	Germline
Cluster 4: 20 genes downregulated by Lh				
tcba0006.g.10	P62993	*grb2*	Growth factor receptor-bound protein 2	–
1RT89F14_D_C07	Q7SYH8	*dab2*	Disabled homolog 2 (*Drosophila*)	–
1RT24E14_C_C07	Q8CI59	*steap3*	Metalloreductase STEAP3	–
tcbk0060.l.19	Q99541	*adfp*	Adipophilin (adipose differentiation-related protein) (ADRP)	Somatic
tcbk0051.k.09	A9JT06	*zgc:110154*	*Danio rerio* Zgc:110154 protein/eukaryotic translation initiation factor 4E	Gonia B
1RT24D22_D_B11	Q7SXT6	*msi2*	RNA-binding protein Musashi homolog 2 (Musashi-2)	Gonia B
1RT24F14_D_C07	–	*rbm28*	RNA-binding protein 28	–
tcbk0051.k.17	F1R444	*prkca*	Protein kinase C α type	Germline
tcba0006.h.12	–	*ehmt2*	Histone-lysine *N*-methyltransferase, H3 lysine-9 specific 3	–
tcbk0045.l.11	Q9UDX4	*sec14l3*	SEC14-like protein 3 (tocopherol-associated protein 2)	–
tcbk0051.n.05	Q7SXY0	*vps4b*	Vacuolar protein sorting-associating protein 4B (yeast)	–
tcbk0015.e.13	Q9P2R6	–	Arginine-glutamic acid dipeptide repeats protein	Somatic
tcbk0054.b.22	–	*pde6h*	Retinal cone rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase subunit gamma	–
1RT24G21_A_D11	Q6NV12	*slc20a1a*	Sodium-dependent phosphate transporter 1	–
tcba0006.i.11	B3DGI9	*timm8b*	Translocase of inner mitochondrial membrane 8 homolog B	–
tcbk0060.h.01	–	*tert*	Telomerase reverse transcriptase	–
tcbk0035.i.22	O42200	*itk*	Tyrosine-protein kinase ITK/TSK	–
1RT24F05_B_C03	–	*capn5*	Calpain-5	–
tcbk0048.l.08	A0N0D8	*creb*	cAMP-responsive element-binding protein	–
tcay0032.c.10	–	*exoc3l2*	Protein 7 transactivated by hepatitis B virus X antigen	Germline

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Some of them were further analyzed by qPCR when they belonged to pathways of interest: *star*, *cebpb*, *inha*, *mmp19*, and *vt1*.

Most importantly, we found a greater number of genes preferentially regulated by one or the other gonadotropin. Hence, Lh regulated more specifically the testicular expression of 175 transcripts (cluster 3 'Up Lh' and cluster 4 'Down Lh'). Furthermore, inside each of these two clusters, we noticed a small subgroup of genes whose regulation by Fsh tended to be the opposite to that by Lh (clusters 3′ and 4′ in Fig. 3 and labeled 'Up Lh, Down Fsh' or 'Down Lh, Up Fsh' in Supplementary information file 1). The genes showing the most differential response between Lh and Fsh are presented in Table 1. These 'Lh specific' genes were involved in different biological processes such as chromatin remodeling, transcription, regulation of translation, signal transduction, cell proliferation, apoptosis, or cell cycle progression. In turn, Fsh modulated preferentially the expression of 82 distinct transcripts annotated 'Up Fsh' or 'Down Fsh' in Supplementary information file 1. Among these 82 genes, those showing the greatest responsiveness to Fsh compared with Lh are listed in Table 2. The examination of the GO terms of the upregulated genes indicated that they were involved in amino acid metabolism, lipid and steroid metabolism, and cation homeostasis processes.

Table 2 Selected genes preferentially regulated by Fsh with the highest differential between the Lh and Fsh responses. These genes from clusters 1 and 6 were identified as differentially regulated by Fsh and Lh in pairwise comparisons (Limma statistical test, FDR <5%)

Clone name	SwissProt/GenBank accession number	Gene symbol	Annotation/ description	Predicted cellular origin
Cluster 1: 22 genes upregulated by Fsh				
tcbk0035.l.03	O95633	*flst3*	Follistatin-related protein 3 precursor	–
tcad0009.j.11	P48307	*tfpi2*	Tissue factor pathway inhibitor 2	Somatic
tcba0001.g.14	Q1L692	*nr1h5*	Farnesoid X receptor FXRβ (fragment)	–
tcay0019.j.04	Q9Y6M5	*slc30a1*	Zinc transporter 1 (ZnT-1) (solute carrier family 30 member 1)	Somatic
1RT41K06_C_F03	–	*mdka*	Pleiotrophin 1 (midkine-related growth factor)	Somatic
tcbk0024.l.04	–	*rrp12*	RRP12-like protein	Gonia B
tcbk0035.k.02	–	*foxo3b*	Forkhead protein FoxO5	Somatic
tcac0003.a.02	Q6F6A1	*ctsl*	Cathepsin L	Somatic
tcbk0048.o.16	Q29VH6	*smtnb*	Smoothelin-b	Somatic
tcay0013.g.08	Q92088	*cyp2m1*	Cytochrome P450 2M1	Somatic
tcba0029.p.03	O95388	*wisp1*	WNT1-inducible-signaling pathway protein 1 precursor (WISP-1)	Somatic
tcab0002.j.23	–	*ldlr*	Vitellogenin receptor (fragment)	Somatic
tcav0002.e.20	–	*dnajc11*	DnaJ homolog subfamily C member 11	–
tcbk0011.i.24	O57656	*gpd1*	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	–
tcay0004.j.03	O95477	*abca1*	ATP-binding cassette sub-family A member 1	Somatic
1RT145G14_C_D07	–	*ggt5*	Gamma-glutamyltransferase 5 precursor	Somatic
1RT153E24_C_C12	A5WUN5	*abr*	Novel protein similar to *H. sapiens* ABR, active BCR-related gene	–
tcay0029.n.03	P15408	*fosl2*	Fos-related antigen 2	Gonia B
1RT102H16_D_D08	A1L222	*krt12*	Keratin 12	Somatic
tcbk0039.m.01	G3NE10	*slc40a1*	Solute carrier family 40 member 1 (ferroportin-1)	Somatic
tcbk0050.a.09	P24468	*nr2f2*	COUP transcription factor 2 (COUP-TF2)	–
tcbk0061.a.18	Q9PVQ9	*hoxc9a*	Homeobox protein Hox-C9	Somatic
Cluster 6: 12 genes downregulated by Fsh				
1RT32D13_B_B07	Q0KFS2	*s100A11*	S100 calcium binding protein (fragment)	Somatic
tcam0001.o.17	Q9TNN8	*onmyUAA-OSU*	MHC class Ia heavy chain	Somatic+gonia
tcay0004.c.04	Q4RMF2	*ttbk2*	Tau-tubulin kinase 2	–
tcad0001.g.06	Q9TNW9	*onmyUBA-SP3*	Major histocompatibility complex class I protein	Somatic
1RT41E09_A_C05	P06396	*gsn*	Gelsolin precursor (actin-depolymerizing factor)	Somatic
tcam0002.m.19	Q8JFQ6	*krt13*	Keratin, type I cytoskeletal 13	Somatic
tcad0007.b.24	G3P658	*rd3*	Protein RD3	Somatic
1RT134E06_C_C03	O14618	*ccs*	Copper chaperone for superoxide dismutase	Somatic
tcay0027.n.15	P02452	*col1a1*	Collagen α-1(I) chain precursor (α-1 type I collagen)	Somatic
tcbk0050.g.24	Q4VBS0	*itga2*	Integrin α-2 precursor	–
tcam0002.m.19	Q7ZTS4	*krt18*	Keratin, type I cytoskeletal 18	Somatic
tcbk0010.d.11	Q564J7	*pum1*	Pumilio homolog 1	Somatic

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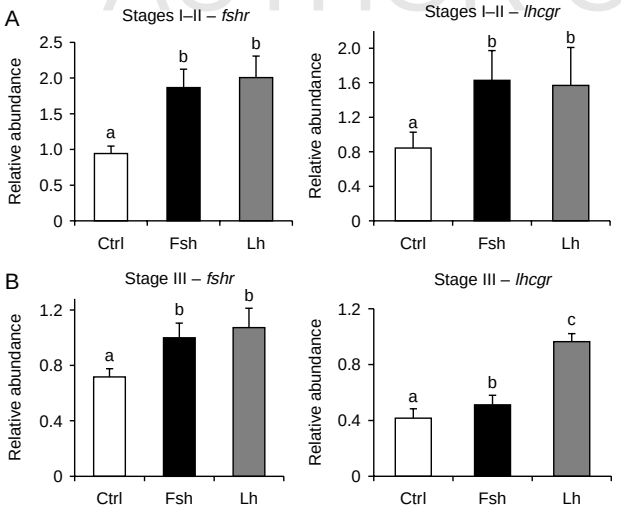

Figure 4
Effect of Fsh and Lh (500 ng/ml) on gonadotropin receptor mRNA levels in the testis explants after 96 h of incubation and according to the testicular stage of development: (A) stages I–II and (B) stage III. Bars represent the mean ± S.D. of five replicates. Different letters indicate that the treatments are significantly different as determined by the non-parametric Mann–Whitney *U* test ($P<0.01$).

These genes encoded nuclear receptors (*nr2f2* and *nr1h5*), proteases (*ctsL* and *ctsS*), transcription factors (*hoxc9a*, *foxo3b*, and *fosl2*), and zinc/iron transporters (*slc30a1* and *slc40a1*). The Fsh-downregulated genes were mostly involved in tissue remodeling and cytoskeleton organization. These genes included *gsn*, *cnn2*, *des*, *actn3*, *krt8*, *krt18*, and *mapre1l*. In the same cluster, we also found several genes that encoded for components of the extracellular matrix including *col1a1*, *col1a2*, *fn1*, *tnc*, and *mmp9*.

In summary, our data show that Fsh and Lh can have either common or independent regulatory effects on gene expression in pubertal trout testis. In stage III, we observed that gonadotropin-regulated transcripts generally displayed similar expression changes as in stages I–II, although less pronounced (Fig. 3).

Gonadotropins regulated remarkable pathways involved in gonadal functions

Taking into account that only Fsh is detected in the blood at the beginning of trout sexual maturation, one major objective of this study was to identify the transcripts that were regulated by Fsh in the testis during the pubertal transition. We therefore focused the data mining on the 226 transcripts (209 non-redundant genes) that were modulated by Fsh either specifically or not specifically (see Supplementary information file 1). All these genes were considered as possible mediators of Fsh actions during spermatogenesis onset. The examination of gene annotation allowed the identification of signaling pathways and processes of relevant interest for the regulation of spermatogenesis. We performed qPCR validation on several of these interesting candidate genes (Figs 4 and 5). We also examined the gonadotropin responsiveness of additional candidate genes that were of particular interest in the pathways analyzed (Table 3 and Fig. 5).

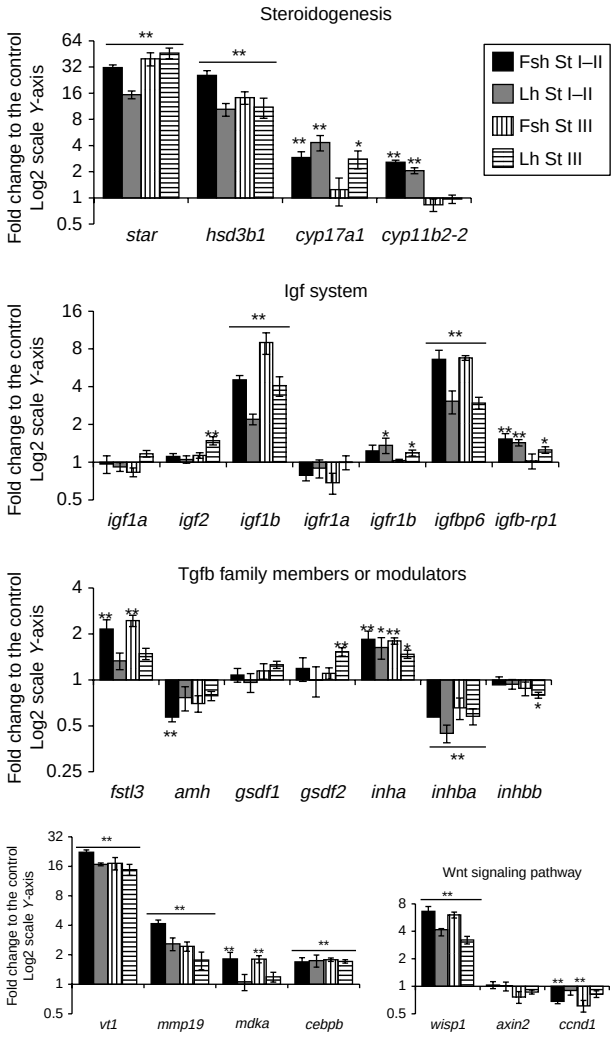

Figure 5
qPCR for the candidate gonadotropin target genes. Testicular tissues were incubated during 96 h in the absence (Ctrl) or presence of Fsh or Lh at 500 ng/ml. After normalization to the reference gene, each individual value was further reported to the mean expression in control samples. Bars represent the mean ± S.E.M. of five to six replicates. The *Y*-axis is log2 scaled and value 1 represents the control level. The asterisks indicate statistical variations of gene expression after the hormonal treatment compared with the control samples (**$P<0.01$, *$P<0.05$).

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Table 3 List of the additional genes studied using qPCR. These genes were either not present on the nylon membrane array used here (or were spotted in a too low amount to be correctly measured) or were not significantly differentially expressed in the array experiment presented here

SwissProt/ GenBank accession number	Gene symbol	Annotation/description	Preferential gonadotropin response	Stage	Predicted cellular origin
Genes absent from microarray					
–	*igf1b/igf3*	Insulin-like growth factor 1b	Up Fsh	I–II and III	Somatic
Q3HWG4	*igfbp6*	Insulin-like growth factor binding protein 6	Up Fsh	I–II and III	–
Q9I8S6	*cyp11b2-2*	Cytochrome P450 11β 2	Up Fsh, Lh	I–II	–
Q71MM8	*fshr*	FSH receptor	Up Fsh, Lh	I–II and III	Somatic
–	*inhbb*	Inhibin β B chain	Up Fsh, Lh	I–II and III	–
–	*gsdf2*	Gonadal somatic cell derived factor 2	Up Lh	III	Somatic
AAC16494	*igfr1b*	Insulin-like growth factor receptor type 1b	Up Lh	I–II and III	–
NM_001165391	*ccnd1*	G1/S-specific cyclin-D1	Down Fsh	I–II and III	–
Q1HG86	*gsdf1*	Gonadal somatic cell derived factor 1	None	–	Somatic
AAC16493	*igfr1a*	Insulin-like growth factor receptor type 1a	None	–	–
Genes present on microarray, badly spotted or not significantly differentially expressed					
P30437	*cyp17a1*	Steroid 17-α-hydroxylase/17,20 lyase	Up Fsh, Lh	I–II and III (Lh)	Somatic
Q71MM9	*lhr*	LH receptor	Up Fsh, Lh	I–II and III	Somatic
P08476	*inhba*	Inhibin β A chain	Up Fsh, Lh	I–II and III	–
Q02815	*igf2*	Insulin-like growth factor 2	Up Lh	III	Somatic
Q02815	*igf1a*	Insulin-like growth factor 1a	None	–	Somatic
P57095	*axin2*	Axin-2	None	–	–

Fsh and Lh modulated gonadotropin receptor transcripts

The control of Fsh and Lh receptor gene expression is an important issue for gonadotropin responsiveness. We observed that Fsh and Lh increased the relative abundance of the two receptor transcripts in testicular tissue after 96 h of incubation. At stages I–II, *fshr* and *lhcgr* were upregulated (about twofold) by both gonadotropins. At stage III, Lh was more efficient than Fsh to upregulate its cognate receptor transcripts *lhcgr* (Fig. 4A and B).

Fsh and Lh stimulated the expression of genes involved in steroidogenesis

The analysis of the microarray data identified several transcripts encoding proteins involved in lipid metabolism, cholesterol efflux, and steroidogenesis such as *star*, *hsd3b1*, *cyp46a1*, and *abca1a*. We measured the differential expression pattern of some of these genes by qPCR (Fig. 5). We also analyzed the expression of two additional transcripts encoding for key enzymes of 11KT synthesis, *cyp17a1* and *cyp11b2-2* (Fig. 5). The two gonadotropins upregulated these transcripts, although differences were observed depending on the gonadotropin used or the stage of development examined. At stages I–II, Fsh was twice more potent than Lh to stimulate *star* and *hsd3b1* expressions, whereas at stage III, Fsh and Lh had similar effects. Lh appeared more efficient than Fsh at upregulating *cyp17a1* expression at both stages. By contrast, Fsh was slightly more active than Lh at stimulating the expression of *cyp11b2-2* at stages I–II and no regulation of this transcript was detected in stage III testis.

Fsh and Lh regulated factors involved in germ cell proliferation and/or differentiation

Growth factor signaling pathways are known to be crucial for the control of spermatogenesis. Because of the suspected role of Igf1 as a mediator of spermatogonial proliferation previously reported in our group, we further investigated the Igf system. In trout, we identified three ligands (Igf1a, Igf1b/Igf3, and Igf2) and two receptors (Igfr1a and Igfr1b). Although the *igf2* gene is duplicated in zebrafish (*igf2a* and *igf2b*), there is no evidence of this duplication in trout and in other known fish genomes. Fsh had a marked upregulatory effect (fourfold) on the expression of the *igf1b* (*igf3*) transcript that encodes for a gonadal paralog of mammalian IGF1. The effect of the gonadotropins was specific to this paralogous gene since no change was observed for *igf1a* and *igf2* transcript expression. Interestingly, the *igfbp6* transcript encoding an Igf-binding protein (Igfbp) was also highly induced (eightfold) by Fsh. The Lh effects on *igf1b* and *igfbp6* were twice lower compared with Fsh. The *igfbp-rP1*

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transcript also showed a moderate (1.5-fold) but significant upregulation by Fsh and Lh at stages I–II and by Lh at stage III. Finally, Lh appeared to have a low but significant stimulatory effect on the Igf receptor isoform *igfr1b*. In conclusion, we demonstrate that important members of the Igf regulatory pathway are targets of the gonadotropins in the fish testis. We show for the first time that the gonad-specific *igf1b* is the only Igf paralog regulated by Fsh.

The regulatory effects of the gonadotropins on the genes encoding for members of the Tgfb family were also investigated. *amh* gene expression was repeatedly downregulated by Fsh, whereas *inha* and *fstl3* were upregulated (Fig. 5). Regarding the transcripts encoding β subunits of activins, we observed that the steady-state level of *inhba* was clearly decreased by both gonadotropins. We extended our investigations to the *gsdf1* and *gsdf2* transcripts. The *gsdf1* gene encodes for a Sertolian factor that stimulates the proliferation of spermatogonia in trout (Sawatari *et al*. 2007). The *gsdf2* gene is a paralog recently described in salmonids only (Lareyre *et al*. 2008). No regulation of these transcripts was observed in the presence of Fsh. Only Lh slightly stimulated *gsdf2* expression at stage III (Fig. 5).

Unexpectedly, the transcript similar to pleiotrophin (*mdka*), encoding a pro-angiogenic cytokine and a growth factor belonging to the neurite growth-promoting factor family, was identified as a specific target of Fsh action. The PCR results confirmed the upregulation of this transcript by Fsh only (about twofold), at both stages of maturation (Fig. 5).

Finally, microarray data analysis showed that genes linked to Wnt signaling, a pathway essential for embryonic development and neurogenesis, were also regulated by the gonadotropins. The *wisp1* and *LOC555552* (*rhou-like*) transcripts were preferentially upregulated by Fsh, whereas the *faf1* transcript was stimulated by the two hormones. By qPCR, we confirmed the strong upregulation of *wisp1* by Fsh (about eightfold). We also explored other known downstream targets of Wnt/β-catenin signaling including *axin2* and *ccnd1*. The *ccnd1* gene was significantly downregulated by Fsh only (about twofold), whereas *axin2* gene expression was not affected by the gonadotropin treatments (Fig. 5 and Supplementary information file 1).

A subset of gonadotropin-regulated genes exhibited relevant expression patterns during the reproductive cycle

The physiological relevance of the *in vitro* regulation of testicular genes by gonadotropins and the predictive cellular origin of the corresponding transcripts were examined by mining the expression data obtained from trout testes collected at different stages of gonadal development and from isolated germ cell fractions, as described in Materials and methods (Rolland *et al*. 2009). Among the gonadotropin-responsive transcripts, 228 were previously identified as differentially expressed in the testis during the reproductive cycle, suggesting that they have a specific role to play during this process. Only 14 genes were predicted as testis or gonad specific (Rolland *et al*. 2009). A first observation is that a majority of these genes ($n=158$, 69.3%) were predicted to be preferentially expressed in somatic cells (Table 4; Supplementary Figure 2, see section on supplementary data given at the end of this article; Supplementary information file 1, column 'testicular expression profile'). This observation is in keeping with the expression of Lhcgr and Fshr in the somatic cells of the testis. The second striking result, illustrated in Fig. 6, is that more than 70% of the somatic genes upregulated by gonadotropins *in vitro* were highly expressed at stage VIII. Some of these genes were involved in pH regulation, ion transport, tissue remodeling, hydration,

Table 4 Predicted cellular origin of the 228 gonadotropin-regulated transcripts deduced from their expression in different stages of testis development and in isolated germ cell fractions enriched in spermatogonia or in more differentiated germ cells (Rolland *et al*. 2009). Data are expressed as a percentage of the total number of genes clustered according to their response to the gonadotropin treatments. The highest values are presented in boldface.

	Response to gonadotropin treatment					
Testicular expression profile	Up Fsh	Up Fsh, Lh	Up Lh	Down Lh	Down Fsh, Lh	Down Fsh
	30	66	38	36	20	38
Somatic expression	**83.3**	**80.3**	**55.3**	38.9	**75.0**	**78.94**
Somatic and spermatogonia	6.7	3.0	5.2	8.3	5.0	10.53
Germ cell expression	10.0	16.7	39.5	**52.8**	20.0	10.53

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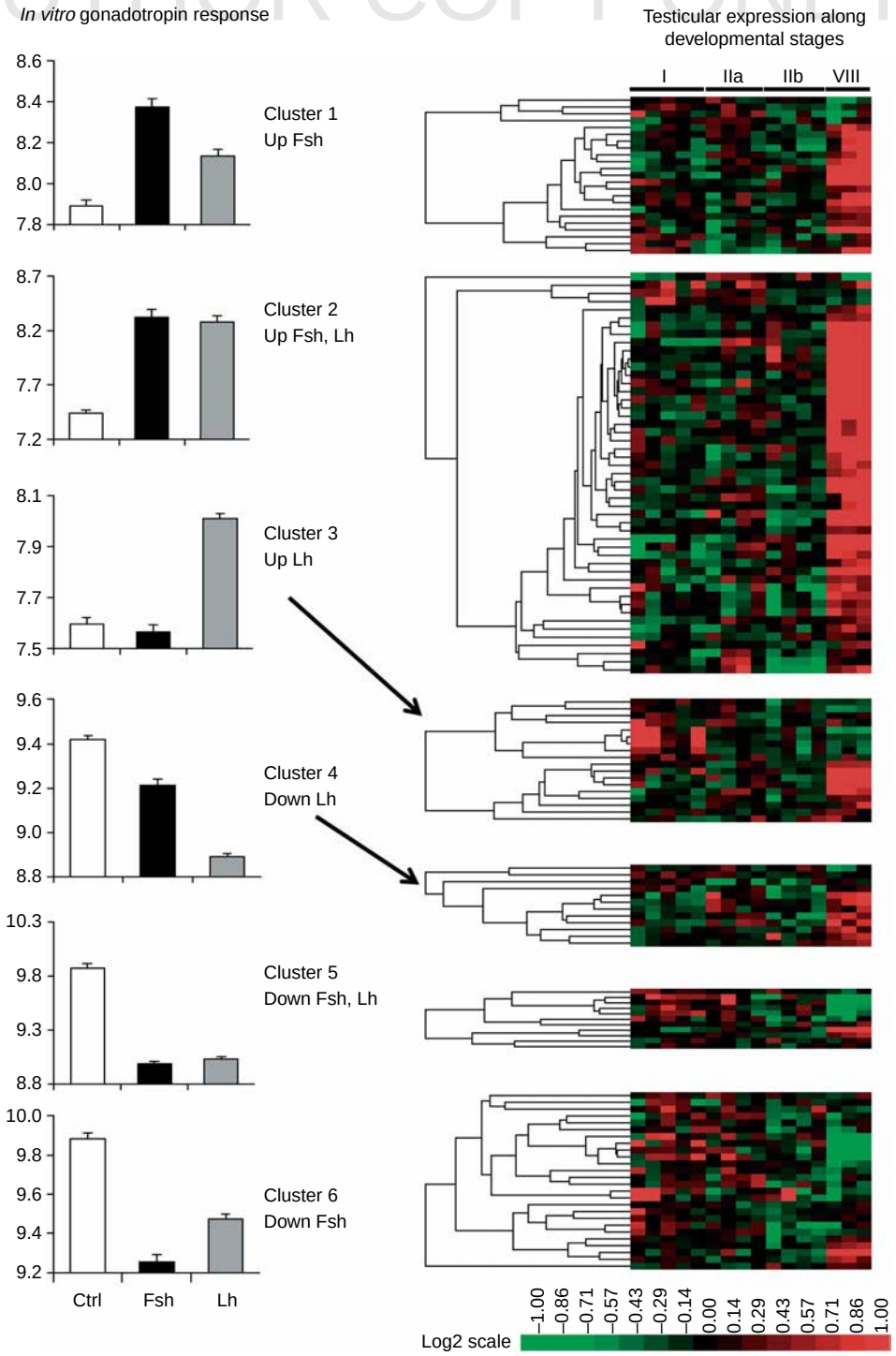

Figure 6

Expression profile of the somatic gonadotropin-regulated genes in the whole testis at different developmental stages. Right: Heatmap representation of the 132 gonadotropin-regulated genes that predicted the origin as somatic. Expression data were obtained from the testes at various developmental stages and from the enriched fractions of isolated germ cells. Roman numerals (I, IIa, IIb, and VIII) indicate the testicular developmental stages (Rolland *et al*. 2009). Log2 transformed signal intensities are shown according to the scale bar. Left: Median expression, at stages I–II, of the 132 gonadotropin-regulated genes in clusters 1–6 that predicted the origin as somatic. Ctrl, no hormonal treatment; Fsh and Lh, treatment with Fsh or Lh (500 ng/ml) during 96 h.

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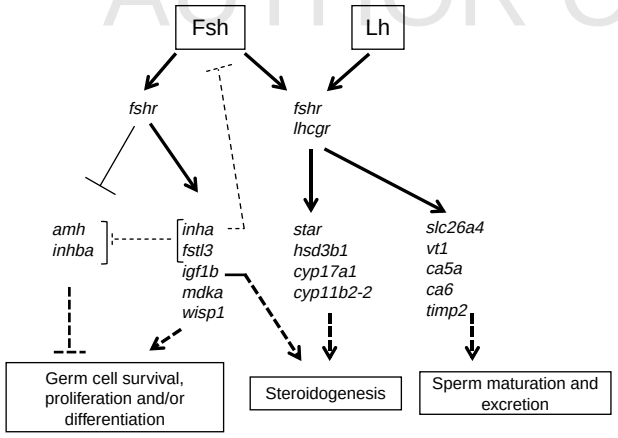

Figure 7
Schematic representation of the predicted function of Fsh- and/or Lh-dependent genes. Fsh and Lh stimulate the gonadotropin receptor genes. Activation of downstream signaling pathways results in the stimulation (arrows) or inhibition (broken line) of the genes. Fsh modulates the genes that have been shown to regulate germ cell survival, proliferation, and/or differentiation. Genes involved in steroidogenesis and sperm maturation/excretion are regulated by both gonadotropins. Besides a role in the paracrine regulation of germ cell proliferation, the positive regulation of *inha* by Fsh (and Lh) suggests that inhibin could exert a negative feedback on the Fsh release as demonstrated in mammals.

and/or smooth muscle contraction (*slc26a4*, *ca5a*, *ca6*, *timp2*, and *vt1*). This suggests that these genes may have a physiological role in sperm maturation or excretion at the end of the reproductive cycle.

Unexpectedly, 56 gonadotropin-regulated transcripts were predicted to be expressed in germ cells. Among the genes regulated by Lh or Fsh, we noticed genes encoding for a cyclin (*ccnb1*), two ATPases involved in proton transport (*atp6v1f* and *atp6ap2*), an apoptotic factor (*ddit4*), and other proteins (*mmp19*, *mina*, *odc1*, and *casp8*) involved in gonadal or germ cell development. Remarkably, Lh affected the expression of a greater number of genes predicted to be expressed in the germ cells compared with Fsh (Table 4).

Discussion

Fsh and Lh gonadotropins modulate the expression of common and distinct genes

Using trout cDNA arrays, we identified the transcripts that were regulated *in vitro* by gonadotropins in testicular explants of pubertal trout after 96 h of exposure.

An important proportion of the transcripts were preferentially up- or downregulated by one or the other gonadotropin. This observation is consistent with the fact that in trout, Fsh and Lh activate preferentially their cognate receptor at the concentration used here (Sambroni *et al*. 2007). Among the genes preferentially regulated by Fsh, we found the transcripts that were previously shown to be expressed in the Sertoli cells, such as *amh* in fish (Skaar *et al*. 2011) and *Wisp1*, *Abca1*, *Smtn*, *Krt18*, *Slc40a1*, *Col1a1*, *Ccnd1*, and *Fstl3* in mice (Chalmel *et al*. 2007). This is in agreement with the fact that the Fsh receptor would be the only gonadotropin receptor expressed in the salmonid Sertoli cells (Miwa *et al*. 1994). The known or putative function of the Fsh-dependent genes will be detailed below.

We also identified 'somatic' genes that were preferentially modulated by Lh, among which several could be of interest concerning the testicular cell fate, such as *pvrl1* encoding a poliovirus receptor-related protein 1 precursor (nectin-1), involved in intercellular adhesion and cell migration, or a tripartite motif protein similar to bloodthirsty, a *trim* gene required for erythropoiesis in zebrafish (Yergeau *et al*. 2005). Many more genes whose function in the gonad remains elusive deserve further examination. Moreover, a noticeable proportion of these genes were predicted to be expressed in germ cells, probably reflecting Lh function in germ cell differentiation or survival. Finally, opposite effects of the two gonadotropins on the expression of a few genes were observed, which reinforces the reality of the differential effects.

More surprisingly in reference to what is described in mammals, our data indicated that one-third of the regulated transcripts were similarly regulated by both Fsh and Lh gonadotropins. Furthermore, among the genes preferentially regulated by Fsh, we found genes known to be regulated by LH in humans or mice as, for example *hsd3b1* or *odc*, the rate-limiting enzyme of polyamine synthesis (Osterman *et al*. 1983). This could be explained by the fact that both receptors, Fshr and Lhcgr, are expressed in the interstitial cells (likely Leydig cells) of the fish testis (see Introduction and Schulz *et al*. (2010)) and could activate common downstream signaling pathways. Furthermore, both receptors were detected in zebrafish seminiferous tubules. Besides, in fish, one could hypothesize that both gonadotropins activate the same receptor type in the target cells. However, as mentioned above, such promiscuous action of Lh on Fsh receptor activity appears unlikely because we previously showed that trout gonadotropins used at 500 ng/ml did not cross-activate their reciprocal receptor (Sambroni *et al*. 2007). Finally, the common effects of the gonadotropins could, at least in part, be mediated through indirect actions resulting from the stimulation of sexual steroids. Indeed,

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we found a stimulation of 11KT production by both gonadotropins, together with a positive regulation of mRNA abundance of several key actors involved in steroid metabolism (*star*, *hsd3b1*, *cyp17a1*, and *cyp11b2-2*). This latter observation is in agreement with previous data obtained *in vitro* in eel (Kazeto *et al*. 2008), zebrafish (Garcia-Lopez *et al*. 2010), or female coho salmon (Luckenbach *et al*. 2011). In fact, several genes regulated by both Fsh and Lh were also found to be similarly regulated by androgens *in vivo* (A D Rolland, A Lardenois, A-S Goupil, J-J Lareyre, F Chalmel, R Houlgatte and F Le Gac, unpublished observations).

Gonadotropin-responsive genes exhibited relevant patterns during the seasonal reproductive cycle

When investigating the expression pattern of the gonadotropin-regulated genes during natural testicular maturation in trout, we found that a majority of them were predicted to be expressed in the somatic cells (about 70%). This observation is in agreement with the expression of the gonadotropin receptor genes in the somatic testicular cells described previously in different teleostean fish species including zebrafish, catfish, eel, and coho salmon (Miwa *et al*. 1994, Ohta *et al*. 2007, Garcia-Lopez *et al*. 2009, 2010).

We further identified a number of genes upregulated *in vitro* by both Fsh and Lh that are also found to be naturally and strongly increased at the end of the reproductive cycle, when both gonadotropins and their receptors reach their maximum secretion and expression levels, respectively (Gomez *et al*. 1999). Such a correlation during the reproductive cycle reinforces the physiological significance of our data. It also shows that, in addition to Lh, Fsh may have important functions during the final stages of sperm maturation and transportation.

In the present study, we show that both gonadotropins upregulate *in vitro* the relative abundance of both *fshr* and *lhcgr* transcripts after a 96-h incubation time. Similar observations were described in mammals. FSH treatment upregulated *Lhcgr* gene expression and steroidogenesis output in the Leydig cells of hypophysectomized immature rats (Vihko *et al*. 1991). In trout, gonadotropins reach their maximum levels just before and during the spawning period at stages VII–VIII (Gomez *et al*. 1999). The positive regulation of the gonadotropin receptor genes observed *in vitro* may explain the dramatic increase in *fshr* and *lhcgr* gene expression described *in vivo* at stage VIII (Sambroni *et al*. 2007). The increased availability of the gonadotropins and their receptors may result in the amplification of the gonadotropin effects on gene expression. In agreement with this assumption, we noticed that a great number of the gonadotropin-responsive genes were highly regulated during the spawning period only (stage VIII).

Molecular and cellular functions underlying the gonadotropin-responsive genes give new insights on the Fsh mechanism of action

The identification of the mediators that relay Fsh actions is of particular relevance in trout since Fsh is the only gonadotropin detected during the pubertal transition and the first stages of testis maturation and is thought to have a major role on early steroidogenesis and spermatogenesis (Gomez *et al*. 1999). However, little is known on the molecular mechanisms of Fsh action. For these reasons, we particularly focus on the genes that were regulated by Fsh.

We showed that Fsh strongly upregulates *igf1b* (*igf3*) and *igfbp6* in the testis. *Igf1b* is a paralog of mammalian *Igf1*, restricted to teleostean fish species and exclusively expressed in the gonads (Wang *et al*. 2008, Zou *et al*. 2009). A recent study showed that hCG increased *igf3* expression in zebrafish ovaries (Irwin & Van Der Kraak 2012). Although another report proposed that *igf1b* might be regulated by the Fsh signaling pathway (Baudiffier *et al*. 2012), the present study is the first demonstration that Fsh upregulates the *igf1b* gene. In our laboratory, we previously demonstrated that rainbow trout spermatogonia harbored type I Igf receptors and that recombinant human or fish IGF1 stimulated their proliferation *in vitro* (Loir & Le Gac 1994, Le Gac *et al*. 1996). In addition, we showed that Fsh stimulated the proliferation of spermatogonia when co-cultured with Sertoli cells (Loir 1999*b*). Altogether, these observations suggest that Fsh increases Igf1b production by the Sertoli cells and, in turn, Igf1b could act as a direct mediator of spermatogonial proliferation. Besides its involvement in the control of spermatogonial proliferation, it has been demonstrated, in tilapia, that recombinant Igf1b upregulated the transcripts encoding for steroidogenic enzymes and transcription factors (*dmrt1*, *nr5a1*, and *foxl2*) involved in their regulation (Li *et al*. 2012). The authors have concluded that Igf1b could be the primary growth factor involved in the regulation of gonadal steroidogenesis. In addition to *igf1b*, we noted that Fsh stimulated the expression of the *igfbp6* transcript. Igfbp are extracellular Igf transport proteins and are considered to be important modulators of Igf actions. Igfbp can either sequester the Igf away from their receptors or they can increase Igf availability through interactions with matrix components located near their

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receptors, thus enhancing Igf activity (Firth & Baxter 2002). Interestingly, no Fsh regulation was observed for the Igf1a and Igf2 ligands. The strong upregulation of *igf1b* and *igfbp6* by Fsh reinforces the idea that the Igf regulatory pathway is a major paracrine pathway that relays Fsh actions on both spermatogenesis and steroidogenesis.

Fsh regulated several genes encoding Tgfb factors, a superfamily known to exert a crucial role in gonadal physiology. They include the *amh*, *inha*, and *inhba* genes. We found that *amh* was downregulated by Fsh in trout. In Japanese eel, Amh (termed spermatogenesis-preventing substance) prevents spermatogonia proliferation and differentiation and is downregulated by 11KT (Miura *et al*. 2002). Recently, in experiments using adult zebrafish testis tissue culture, Amh was shown to inhibit spermatogenesis and was downregulated by Fsh (Skaar *et al*. 2011). Interestingly, during the natural trout reproductive cycle, *amh* mRNA levels are abundant in Sertoli cells in stages I–II and become hardly detectable towards spawning (Rolland *et al*. 2009), when gonadotropins and androgen plasma levels are high (Gomez *et al*. 1999). Thus, the Fsh-induced downregulation of *amh* observed in the present study *in vitro* could reflect the physiological activity of Fsh during the maturation of the testis. In conclusion, there is accumulating evidence that Fsh suppresses the inhibitory action of Amh, allowing the commitment of germ cells to spermatogenesis.

The activins A and B are homodimers of the β subunits βa and βb. These subunits are encoded by the *inhba* and *inhbb* genes, respectively. The inhibins are heterodimers of an α subunit (encoded by *inha*) and one of the β subunits shared with the activins. Our data showed that the transcripts encoding for the three subunits (*inha*, *inhba*, and *inhbb*) are regulated independently by Fsh and Lh. *inhba* was downregulated by both gonadotropins, whereas *inhbb* was moderately downregulated by Lh. In rodents, numerous *in vivo* and *in vitro* studies have demonstrated the importance of activin A and its regulators (inhibin and follistatin) in the control of spermatogenesis (de Kretser *et al*. 2004, Barakat *et al*. 2008, 2012). In fetal and postnatal testis, activin A promotes both Sertoli cell and gonocyte proliferation but hampers the differentiation of gonocytes in spermatogonia (Mithraprabhu *et al*. 2010, Fan *et al*. 2012). Meehan *et al*. (2000) hypothesized that a reduction of activin A bioactivity is required for the onset of spermatogenesis in the early postnatal testis, to drive the differentiation of spermatogonial germ cells. Taken together, these observations suggest that Fsh could stimulate spermatogonial differentiation through the inhibition of the Ba subunit.

Interestingly, the *inha* transcript was upregulated by Fsh and Lh. In addition, follistatin-like 3 (Fstl-3) transcripts were increased by Fsh treatment. Fstl-3 is predominantly expressed in mouse testis (Xia *et al*. 2004) and, similar to inhibin, is able to antagonize the actions of activins (Schneyer *et al*. 2004). In summary, our data suggest that Fsh actions converge to an inhibition of both Amh and activin signaling to finely adjust the balance between germ cell proliferation and differentiation.

Gsdf is another teleostean specific member of the Tgfb family predominantly expressed in the Sertoli cells surrounding spermatogonia (Sawatari *et al*. 2007, Gautier *et al*. 2011). This factor has been involved in the proliferation of primordial germ cells (Sawatari *et al*. 2007), early testicular differentiation (Shibata *et al*. 2010), and sex determination in one fish species (Myosho *et al*. 2012). Surprisingly, our data indicate that Fsh did not regulate *gsdf* gene expression. In agreement with our results, *gsdf* gene expression was not regulated by Fsh in coho salmon ovary (Luckenbach *et al*. 2011) or in the mature male testis of zebrafish (Garcia-Lopez *et al*. 2010). In summary, although Gsdf was proposed as a stimulatory factor of germ cell proliferation, there is no evidence that its expression is regulated by Fsh in fish.

Pleiotrophin mRNA levels were exclusively upregulated by Fsh. Pleiotrophin is a secreted heparin-binding cytokine that regulates numerous functions, including mitogenesis, angiogenesis, and cell differentiation. Its role in the testis is poorly understood, except that a dominant negative mutation led to an increase in germ cell apoptosis in mice (Zhang *et al*. 1999). Considering pleiotrophin (*mdka*) functions, this growth factor specifically regulated by Fsh appears as a new strong candidate gene regulating germ cell survival in trout.

Our study showed that Fsh regulates the steady-state level of several mRNAs connected with the Wnt pathway (*wisp1*, *LOC555552*, and *faf1*). The activation of the Wnt pathway was involved in the proliferation and self-renewal of mouse and human spermatogonia (Boyer *et al*. 2008, Tanwar *et al*. 2010). In the present study, we observed that Fsh negatively regulated the *ccnd1* gene. Interestingly, a similar negative regulation has also been described in mice (Meachem *et al*. 2005). In this species, the expression of cyclin D1 is detected only in proliferating gonocytes and spermatogonia, suggesting a role in the G(1)/S phase transition (Beumer *et al*. 2000). One could hypothesize that the high levels of Fsh observed at stage VIII could suppress cyclin D1 expression and maintain the germ stem cells in a quiescent status.

Interestingly, the expression of genes encoding components or remodeling enzymes of the extracellular matrix

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was also found to be regulated by Fsh (*mmp19*, *mmp9*, *timp2*). In the gilthead seabream, *mmp9* and *timp2* are expressed in the testis and are differentially expressed during the reproductive cycle, in association with testis remodeling (Chaves-Pozo *et al*. 2008). In rats, metalloproteinases are expressed in Sertoli cells and have been involved in the changes in the Sertoli cell cytoskeleton elicited by FSH (Longin & Le Magueresse-Battistoni 2002). Metalloproteinases could also participate in the regulation of the Sertoli cell junction dynamic that is important for the function of the hemato–testicular barrier (Siu *et al*. 2003). This suggests that, in addition to the stimulation or inhibition of paracrine growth factors, Fsh could be involved in the dramatic remodeling of the testicular cystic structure that occurs during the pubertal transition. Further investigations will be required to determine whether the components of the extracellular matrix or their modifications are involved in the differentiation of Sertoli cells and/or germ cells.

In conclusion, we showed for the first time on a large scale that Lh and Fsh have similar but also preferential or specific regulatory effects on numerous genes in the fish testis. We confirm that both gonadotropins stimulate the production of androgens and the expression of several steroidogenic enzyme genes. Further investigations will be required to determine to what extent the similar effects of Fsh and Lh are mediated through the stimulation of sexual steroid production. Our data suggest that Fsh acts through multiple convergent regulatory pathways to finely adjust the balance between germ cell quiescence, proliferation, and/or differentiation. These regulatory pathways include the stimulation of the Igf system and the inhibition of Amh and activin pathways. Finally, the present study identifies new gonadotropin-responsive pathways or genes and provides a solid foundation for further studies on the physiological relevance of Fsh and Lh in fish models.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-12-0197.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
E S performed the tissue culture experiments, the microarray data analyses, and the real-time PCR validations, and drafted the manuscript. F L G and J-J L supervised the study. E S, A R, and F L G participated in the analyses of the microarray data, and F L G and J-J L polished the manuscript. All authors read and approved the final version of the manuscript.

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References

Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG & Charlton HM 2000 The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141** 1795–1803. (doi:10.1210/en.141.5.1795)

Abel MH, Baban D, Lee S, Charlton HM & O'Shaughnessy PJ 2009 Effects of FSH on testicular mRNA transcript levels in the hypogonadal mouse. *Journal of Molecular Endocrinology* **42** 291–303. (doi:10.1677/JME-08-0107)

Alam MA, Kobayashi Y, Hirai T & Nakamura M 2010 Isolation, characterization and expression analyses of FSH receptor in protogynous grouper. *Comparative Biochemistry and Physiology. Part A: Molecular and Integrative Physiology* **156** 364–371. (doi:10.1016/j.cbpa.2010.03.001)

Andersson E, Nijenhuis W, Male R, Swanson P, Bogerd J, Taranger GL & Schulz RW 2009 Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. *General and Comparative Endocrinology* **163** 329–339. (doi:10.1016/j.ygcen.2009.05.001)

Barakat B, O'Connor AE, Gold E, de Kretser DM & Loveland KL 2008 Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction* **136** 345–359. (doi:10.1530/REP-08-0140)

Barakat B, Itman C, Mendis SH & Loveland KL 2012 Activins and inhibins in mammalian testis development: new models, new insights. *Molecular and Cellular Endocrinology* **359** 66–77. (doi:10.1016/j.mce.2012.02.018)

Baudiffier D, Hinfray N, Vosges M, Creusot N, Chadili E, Porcher JM, Schulz RW & Brion F 2012 A critical role of follicle-stimulating hormone (Fsh) in mediating the effect of clotrimazole on testicular steroidogenesis in adult zebrafish. *Toxicology* **298** 30–39. (doi:10.1016/j.tox.2012.04.012)

Beumer TL, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG 2000 Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. *Biology of Reproduction* **63** 1893–1898. (doi:10.1095/biolreprod63.6.1893)

Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ & Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **64** 1633–1643. (doi:10.1095/biolreprod64.6.1633)

Boyer A, Hermo L, Paquet Mn, Robaire B & Boerboom D 2008 Seminiferous tubule degeneration and infertility in mice with sustained activation of WNT/CTNNB1 signaling in Sertoli cells. *Biology of Reproduction* **79** 475–485. (doi:10.1095/biolreprod.108.068627)

Published by Bioscientifica Ltd.

Chalmel F & Primig M 2008 The annotation, mapping, expression and network (AMEN) suite of tools for molecular systems biology. *BMC Bioinformatics* **9** 86. (doi:10.1186/1471-2105-9-86)

Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, Gattiker A, Moore J, Patard JJ, Wolgemuth DJ & Jegou B 2007 The conserved transcriptome in human and rodent male gametogenesis. *PNAS* **104** 8346–8351. (doi:10.1073/pnas.0701883104)

Chaves-Pozo E, Castillo-Briceno P, Garcia-Alcazar A, Meseguer J, Mulero V & Garcia-Ayala A 2008 A role for matrix metalloproteinases in granulocyte infiltration and testicular remodelation in a seasonal breeding teleost. *Molecular Immunology* **45** 2820–2830. (doi:10.1016/j.molimm.2008.01.031)

Fan YS, Hu YJ & Yang WX 2012 TGF-B superfamily: how does it regulate testis development. *Molecular Biology Reports* **39** 4727–4741. (doi:10.1007/s11033-011-1265-5)

Firth SM & Baxter RC 2002 Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews* **23** 824–854. (doi:10.1210/er.2001-0033)

Fostier A, Billard R, Breton B, Legendre M & Marlot S 1982 Plasma 11-oxotestosterone and gonadotropin during the beginning of spermiation in rainbow-trout (*Salmo-Gairdneri* R). *General and Comparative Endocrinology* **46** 428–434. (doi:10.1016/0016-6480(82)90096-X)

Garcia-Lopez A, Bogerd J, Granneman JCM, van Dijk W, Trant JM, Taranger GL & Schulz RW 2009 Leydig cells express follicle-stimulating hormone receptors in African catfish. *Endocrinology* **150** 357–365. (doi:10.1210/en.2008-0447)

Garcia-Lopez A, de Jonge H, Nobrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J & Schulz RW 2010 Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151** 2349–2360. (doi:10.1210/en.2009-1227)

Gautier A, Le Gac F & Lareyre JJ 2011 The gsdf gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes. *Gene* **472** 7–17. (doi:10.1016/j.gene.2010.10.014)

Gomez JM, Weil C, Ollitrault M, Le Bail PY & Le Gac F 1999 Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **113** 413–428. (doi:10.1006/gcen.1998.7222)

Huhtaniemi I 2006 Mutations along the pituitary–gonadal axis affecting sexual maturation: novel information from transgenic and knockout mice. *Molecular and Cellular Endocrinology* **254–255** 84–90. (doi:10.1016/j.mce.2006.04.015)

Irwin DA & Van Der Kraak G 2012 Regulation and actions of insulin-like growth factors in the ovary of zebrafish (*Danio rerio*). *General and Comparative Endocrinology* **177** 187–194. (doi:10.1016/j.ygcen.2012.03.006)

Kazeto Y, Kohara M, Miura T, Miura C, Yamaguchi S, Trant JM, Adachi S & Yamauchi K 2008 Japanese eel follicle-stimulating hormone (fsh) and luteinizing hormone (lh): production of biologically active recombinant fsh and lh by *Drosophila* s2 cells and their differential actions on the reproductive biology. *Biology of Reproduction* **79** 938–946. (doi:10.1095/biolreprod.108.070052)

de Kretser DM, Buzzard JJ, Okuma Y, O'Connor AE, Hayashi T, Lin SY, Morrison JR, Loveland KL & Hedger MP 2004 The role of activin, follistatin and inhibin in testicular physiology. *Molecular and Cellular Endocrinology* **225** 57–64. (doi:10.1016/j.mce.2004.07.008)

Kumar TR 2005 What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? *Reproduction* **130** 293–302. (doi:10.1530/rep.1.00660)

Lareyre JJ, Ricordel MJ, Mahe S, Goupil AS, Vizziano D, Bobe J, Guiguen Y & Le Gac F 2008 Two new TGFβ members are restricted to the gonad and differentially expressed during sex differentiation and gametogenesis in trout. *Cybium* **32** 202.

Le Gac F, Loir M, Le Bail PY & Ollitrault M 1996 Insulin-like growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. *Molecular Reproduction and Development* **44** 23–35. (doi:10.1002/(SICI)1098-2795(199605)44:1<23::AID-MRD3>3.0.CO;2-V)

Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X & Rao CV 2001 Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Molecular Endocrinology* **15** 184–200. (doi:10.1210/me.15.1.184)

Levavi-Sivan B, Bogerd J, Mananos EL, Gomez A & Lareyre JJ 2010 Perspectives on fish gonadotropins and their receptors. *General and Comparative Endocrinology* **165** 412–437. (doi:10.1016/j.ygcen.2009.07.019)

Li M, Wu F, Gu Y, Wang T, Wang H, Yang S, Sun Y, Zhou L, Huang X, Jiao B *et al*. 2012 Insulin-like growth factor 3 regulates expression of genes encoding steroidogenic enzymes and key transcription factors in the nile tilapia gonad. *Biology of Reproduction* **86** 163. (doi:10.1095/biolreprod.111.096248)

Loir M 1999*a* Spermatogonia of rainbow trout: I. Morphological characterization, mitotic activity, and survival in primary cultures of testicular cells. *Molecular Reproduction and Development* **53** 422–433. (doi:10.1002/(SICI)1098-2795(199908)53:4<422::AID-MRD8>3.0.CO;2-V)

Loir M 1999*b* Spermatogonia of rainbow trout: II. *in vitro* study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Molecular Reproduction and Development* **53** 434–442. (doi:10.1002/(SICI)1098-2795(199908)53:4<434::AID-MRD9>3.0.CO;2-L)

Loir M & Le Gac F 1994 Insulin-like growth factor-I and -II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes. *Biology of Reproduction* **51** 1154–1163. (doi:10.1095/biolreprod51.6.1154)

Longin J & Le Magueresse-Battistoni B 2002 Evidence that MMP-2 and TIMP-2 are at play in the FSH-induced changes in Sertoli cells. *Molecular and Cellular Endocrinology* **189** 25–35. (doi:10.1016/S0303-7207(01)00756-0)

Lopez F, Rougemont J, Loriod B, Bourgeois A, Loi L, Bertucci F, Hingamp P, Houlgatte R & Granjeaud S 2004 Feature extraction and signal processing for nylon DNA microarrays. *BMC Genomics* **5** 38. (doi:10.1186/1471-2164-5-38)

Luckenbach JA, Dickey JT & Swanson P 2011 Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors *in vitro* during early secondary oocyte growth in coho salmon. *General and Comparative Endocrinology* **171** 52–63. (doi:10.1016/j.ygcen.2010.12.016)

McLean DJ, Friel PJ, Pouchnik D & Griswold MD 2002 Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. *Molecular Endocrinology* **16** 2780–2792. (doi:10.1210/me.2002-0059)

Meachem SJ, Ruwanpura SM, Ziolkowski J, Ague JM, Skinner MK & Loveland KL 2005 Developmentally distinct *in vivo* effects of FSH on proliferation and apoptosis during testis maturation. *Journal of Endocrinology* **186** 429–446. (doi:10.1677/joe.1.06121)

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM & Loveland KL 2000 Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. *Developmental Biology* **220** 225–237. (doi:10.1006/dbio.2000.9625)

Mithraprabhu S, Mendis S, Meachem SJ, Tubino L, Matzuk MM, Brown CW & Loveland KL 2010 Activin bioactivity affects germ cell differentiation in the postnatal mouse testis *in vivo*. *Biology of Reproduction* **82** 980–990. (doi:10.1095/biolreprod.109.079855)

Miura T, Miura C, Konda Y & Yamauchi K 2002 Spermatogenesis-preventing substance in Japanese eel. *Development* **129** 2689–2697.

Miwa S, Yan L & Swanson P 1994 Localization of two gonadotropin receptors in the salmon gonad by *in vitro* ligand autoradiography. *Biology of Reproduction* **50** 629–642. (doi:10.1095/biolreprod50.3.629)

http://www.jme.endocrinology-journals.org
DOI: 10.1530/JME-12-0197

Published by Bioscientifica Ltd.

Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A, Naruse K, Hamaguchi S & Sakaizumi M 2012 Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. *Genetics* **191** 163–170. (doi:10.1534/genetics.111.137497)

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H & Nagahama Y 1999 The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications* **265** 366–371. (doi:10.1006/bbrc.1999.1700)

Ohta T, Miyake H, Miura C, Kamei H, Aida K & Miura T 2007 Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. *Biology of Reproduction* **77** 970–977. (doi:10.1095/biolreprod.107.062299)

Osterman J, Barnett D, Murono EP, Lin T & Nankin HR 1983 Stimulation of ornithine decarboxylase activity by luteinizing hormone in rat testicular interstitial cells *in vitro* is age dependent. *Life Sciences* **33** 353–357. (doi:10.1016/S0024-3205(83)80008-3)

Planas JV & Swanson P 1995 Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) *in vitro*. *Biology of Reproduction* **52** 697–704. (doi:10.1095/biolreprod52.3.697)

Prat F, Sumpter JP & Tyler CR 1996 Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biology of Reproduction* **54** 1375–1382. (doi:10.1095/biolreprod54.6.1375)

Rescan PY, Montfort J, Ralliere C, Le Cam A, Esquerre D & Hugot K 2007 Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. *BMC Genomics* **8** 438. (doi:10.1186/1471-2164-8-438)

Rolland A, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ, Esquerre D, Hugot K, Houlgatte R, Chalmel F & Le Gac F 2009 Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* **10** 546. (doi:10.1186/1471-2164-10-546)

Sadate-Ngatchou PI, Pouchnik DJ & Griswold MD 2004 Follicle-stimulating hormone induced changes in gene expression of murine testis. *Molecular Endocrinology* **18** 2805–2816. (doi:10.1210/me.2003-0203)

Sambroni E, Le Gac F, Breton B & Lareyre JJ 2007 Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. *Journal of Endocrinology* **195** 213–228. (doi:10.1677/JOE-06-0122)

Sawatari E, Shikina S, Takeuchi T & Yoshizaki G 2007 A novel transforming growth factor-β superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). *Developmental Biology* **301** 266–275. (doi:10.1016/j.ydbio.2006.10.001)

Schneyer A, Sidis Y, Xia Y, Saito S, Re Ed, Lin HY & Keutmann H 2004 Differential actions of follistatin and follistatin-like 3. *Molecular and Cellular Endocrinology* **225** 25–28. (doi:10.1016/j.mce.2004.02.009)

Schulz RW, Menting S, Bogerd J, Franca LR, Vilela DAR & Godinho HP 2005 Sertoli cell proliferation in the adult testis-evidence from two fish species belonging to different orders. *Biology of Reproduction* **73** 891–898. (doi:10.1095/biolreprod.105.039891)

Schulz RW, de Franca LR, Lareyre JJ, LeGac F, Chiarini-Garcia H, Nobrega RH & Miura T 2010 Spermatogenesis in fish. *General and Comparative Endocrinology* **165** 390–411. (doi:10.1016/j.ygcen.2009.02.013)

Shibata Y, Paul-Prasanth B, Suzuki A, Usami T, Nakamoto M, Matsuda M & Nagahama Y 2010 Expression of gonadal soma derived factor (GSDF) is spatially and temporally correlated with early testicular differentiation in medaka. *Gene Expression Patterns* **10** 283–289. (doi:10.1016/j.gep.2010.06.005)

Siu MKY, Lee WM & Cheng CY 2003 The interplay of collagen IV, tumor necrosis factor-α, gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloproteases-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. *Endocrinology* **144** 371–387. (doi:10.1210/en.2002-220786)

Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW & Male R 2011 Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* **152** 3527–3540. (doi:10.1210/en.2010-1469)

Smyth GK, Michaud J & Scott HS 2005 Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* **21** 2067–2075. (doi:10.1093/bioinformatics/bti270)

So WK, Kwok HF & Ge W 2005 Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits – their spatial–temporal expression patterns and receptor specificity. *Biology of Reproduction* **72** 1382–1396. (doi:10.1095/biolreprod.104.038216)

Tanwar PS, Kaneko-Tarui T, Zhang L, Rani P, Taketo MM & Teixeira J 2010 Constitutive WNT/β-catenin signaling in murine Sertoli cells disrupts their differentiation and ability to support spermatogenesis. *Biology of Reproduction* **82** 422–432. (doi:10.1095/biolreprod.109.079335)

Vihko KK, Lapolt PS, Nishimori K & Hsueh AJW 1991 Stimulatory effects of recombinant follicle-stimulating hormone on Leydig cell function and spermatogenesis in immature hypophysectomized rats. *Endocrinology* **129** 1926–1932. (doi:10.1210/endo-129-4-1926)

Vischer HF & Bogerd J 2003 Cloning and functional characterization of a gonadal luteinizing hormone receptor complementary DNA from the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **68** 262–271. (doi:10.1095/biolreprod.102.004515)

Vischer HF, Granneman JC, Linskens MH, Schulz RW & Bogerd J 2003 Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. *Journal of Molecular Endocrinology* **31** 133–140. (doi:10.1677/jme.0.0310133)

Wang DS, Jiao B, Hu C, Huang X, Liu Z & Cheng CHK 2008 Discovery of a gonad-specific IGF subtype in teleost. *Biochemical and Biophysical Research Communications* **367** 336–341. (doi:10.1016/j.bbrc.2007.12.136)

Xia Y, Sidis Y & Schneyer A 2004 Overexpression of follistatin-like 3 in gonads causes defects in gonadal development and function in transgenic mice. *Molecular Endocrinology* **18** 979–994. (doi:10.1210/me.2003-0364)

Yan L, Swanson P & Dickhoff WW 1992 A two-receptor model for salmon gonadotropins (GTH I and GTH II). *Biology of Reproduction* **47** 418–427. (doi:10.1095/biolreprod47.3.418)

Yergeau DA, Cornell CN, Parker SK, Zhou Y & Detrich HW III 2005 bloodthirsty, an RBCC/TRIM, gene required for erythropoiesis in zebrafish. *Developmental Biology* **283** 97–112. (doi:10.1016/j.ydbio.2005.04.006)

Zhang N, Yeh HJ, Zhong R, Li YS & Deuel TF 1999 A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice. *PNAS* **96** 6734–6738. (doi:10.1073/pnas.96.12.6734)

Zhang FP, Poutanen M, Wilbertz J & Huhtaniemi I 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Molecular Endocrinology* **15** 172–183. (doi:10.1210/me.15.1.172)

Zou S, Kamei H, Modi Z & Duan C 2009 Zebrafish IGF genes: gene duplication, conservation and divergence, and novel roles in midline and notochord development. *PLoS ONE* **4** e7026. (doi:10.1371/journal.pone.0007026)

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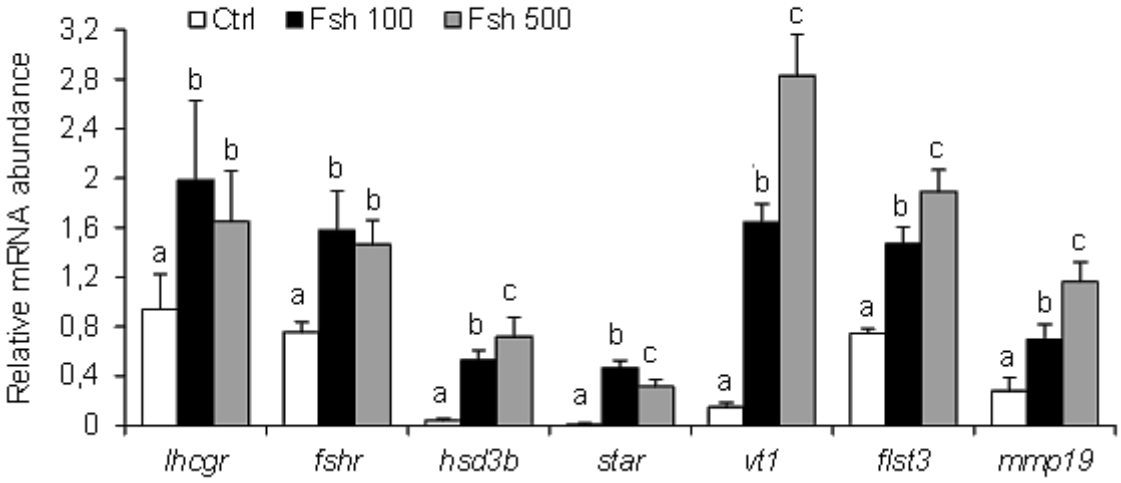
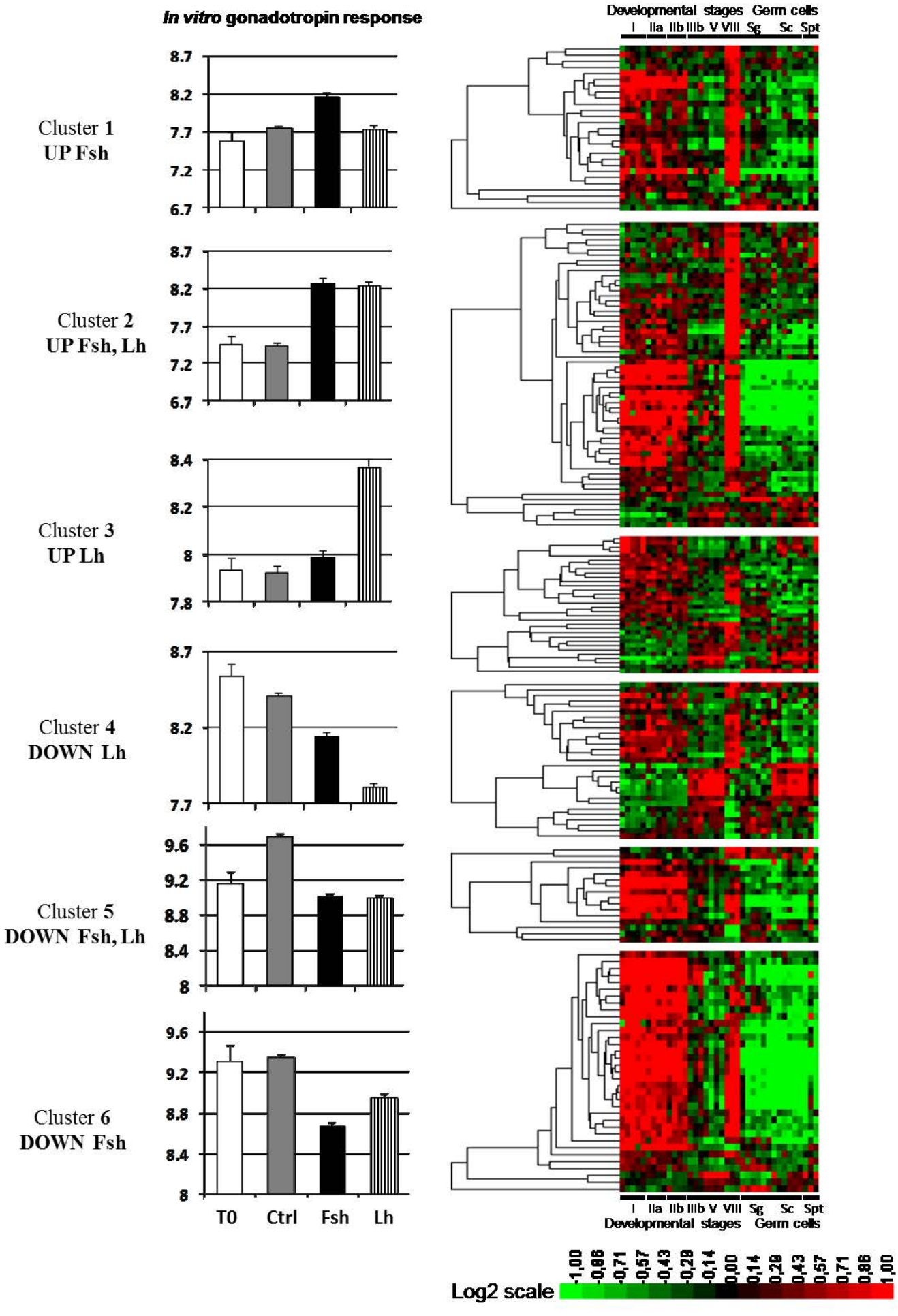
Supplementary table 1: Sequences of primers used for q-PCR experiments.

Gene symbol	Acc number	Forward primer	Reverse primer
rs15 (ref)	ACO08621	CCTGGGGGAGTTCTCTATCACCT	GGGATGAAACGGGAAGAATGTGT
fshr	AF439405	TCAGTCACCTGACGATCTGCAA	TCCTGCAGGTCCAGCAGAAACG
lhr	AF439404	CTTCTCAACCTCAATGAAATCTTC	GGATATACTCAGATAACGCAGCTT
igf1a	M95183	TGGACACGCTGCAGTTTGTGTGT	CACTCGTCCACAATACCACGGTT
igf1b	CX025953	GTGTGGAGACCGTGGATTTT	CACAATTCCCTTCCCTCTCA
igf2	M95184	CGGCAGAAACGCTATGTGGA	TGCTGGTTGGCCTACTGAAA
igfr1a	AF062499	AGAGATAGACGACGCCTCCTA	CACCAAATAGATCCCTACGT
igfr1b	AY100460	CCTAAATCTGTAGGAGACCTGGAG	GGTTAGCCACGCCAAATAGATCC
igfbp6	DQ190459	GCTCAATAGTGTTCTGCGTGG	CTTGGAGGAACGACACTGCTT
igfbrp1	DQ146965	GCTCCGATGGAGTGACCTATA	ACAATGACAGGTGCTGTTGCG
star	AB047032	GAGTTGTTAGGGCAGAGAAC	CAACCCTTTAAATCTATGCTTA
hsd3b	S72665	TACAGTGCCTGGAAGAGATCAGA	ACCCTGTGAAGCTCACTGTATAA
cyp17a1	X65800	ACAGTAACCACAGACCTGTTG	CCTACAGATAATCTTCTCGAT
cyp11b2	AF217273	CTGGGACATGTGTCCAGGCA	CTGGATCCTGAAACACGTCA
flst3	NM_001160487	ACCGCTGAAGTCCGAGTTGC	GCAGGTGGCTCTGTGGAGGT
amh	Q5XZF0	GGGAATAACCATGCTATCCTGCTTAA	CTCCACCACCTTGAGGTCCTCATAGT
inha	Q9DED3	CCAGCTCTGACTCTACCTGTGAT	CCTGGTTGTCGAGGGAGGATTG
inhba	D88463	AGGGCAAGGTGAACATACAG	CCTCGTGTCCACCATCTTCTC
inhbb	AB044566	GTTTCGCAGAGACAGATGAG	GTCACATACAGGTGCTGGTT
gsdf1	NM_001124579	TCACAACCCTCAAGCGCTAGA	ATGAGAGCTCTGCTGTTATTG
gsdf2	Unpublished	ACTTGGTCCCAGTGCTCTACCTTG	AAGTATGGCGATCTGGGTTGATG
vt1	CA375992	GAGGCTGGAGGAAGAGTGTG	TTCTGTTTGCTGGGTGACTG
mmp19	BX081049	AGTTCTGAGGGAGTGTGTGG	TGTTGTGAGGGATAGGAAGG
ptn	AF149802	CAGTGTTCTTGGTCTGCCTAA	CTTGGTCTTGACTCCAGTTGA
cebpb	AAQ18795	GTCTCAAGAGGACCCAAGAATG	GTGGACTGGAGGAAGACGAG
wisp1	BX868220	TTCAACTCAACAGGAAGAGC	AACTCAAGGAGGGTCAAGAT
axin2	BX320381	GTGGATCCTGGAGAGTGACC	GCCACCATACGCCTTCTTAG
ccnd1	CA374091	GTCCCTTTAACTGCAGAGAAGT	ATCGTGAGGTGTTACTGATGCT



Supplementary figure 1: Dose response experiment. Effect of two Fsh concentrations on the steady-state level of 7 mRNA measured by q-PCR in testis tissue incubated during 96h without (Ctrl) or with Fsh at 100ng/mL (Fsh 100) and at 500 ng/mL (Fsh 500). Bars represent the mean ± SD of 5 replicates.


In vitro gonadotropin response
Developmental stages Germ cells
I IIa IIb IIIb V VIII Sg Sc Spt
Cluster 1
UP Fsh
Cluster 2
UP Fsh, Lh
Cluster 3
UP Lh
Cluster 4
DOWN Lh
Cluster 5
DOWN Fsh, Lh
Cluster 6
DOWN Fsh
8.7
8.2
7.7
7.2
6.7
8.4
8
7.8
9.6
9.2
8.8
8.4
T0 Ctrl Fsh Lh
Log2 scale
-1,00 -0,86 -0,71 -0,57 -0,43 -0,29 -0,14 0,00 0,14 0,29 0,43 0,57 0,71 0,86 1,00

Supplementary figure 2: Expression profile of gonadotropin-regulated genes in the whole testis at different developmental stages and in isolated germ cells.
Right panel: Heatmap representation of the 228 gonadotropin-regulated genes that also display differential expression during trout spermatogenesis. Expression data were obtained from testes at various developmental stages and from enriched fractions of isolated germ cells. Roman numerals (I-V and VIII) indicate testicular developmental stages. Sg, Sc and Spt correspond to isolated germ cell populations enriched in spermatogonia, spermatocytes and spermatids, respectively (Rolland *et al.* 2009). Each line represents the expression signal of a clone and each column is a sample. Log-2 transformed signal intensities are shown according to the scale bar.
Left panel: Histograms showing median expression profiles, at stage I-II, for each cluster of the gonadotropin-regulated genes. Ctrl: no hormonal treatment, Fsh and Lh: treatment with Fsh or Lh (500 ng/mL) during 96 hours.

Supplementary file 1: Gonadotropin-responsive genes in the trout testis.

The searchable excel file contains quantile-quantile normalized expression data (Log-2 transformed), annotations, and information about "i*n vitro* gonadotropin response" at each studied stage, and about "Testicular expression profile" for the 403 gonadotropin-responsive clones. Annotation provided contains the "Clone Name", the fish ortholog "Gene Symbol" and "Gene name", according to the following species availability: Gasteosteus aculeatus, Danio rerio, Oryzias latipes or Takifugu rubripes), the best protein homologs and associated GeneOntology terms and IDs ("Biological process", Molecular function" and "Cellular component").

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Fsh Controls Gene Expression in Fish both Independently of and through Steroid Mediation

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Abstract

The mechanisms and the mediators relaying Fsh action on testicular functions are poorly understood. Unlike in mammals, in fish both gonadotropins (Fsh and Lh) are able to efficiently stimulate steroidogenesis, likely through a direct interaction with their cognate receptors present on the Leydig cells. In this context, it is crucial to understand if Fsh effects are mediated through the production of steroids. To address this issue we performed transcriptome studies after *in vitro* incubations of rainbow trout testis explants in the presence of Fsh alone or in combination with trilostane, an inhibitor of Δ4-steroidogenesis. Trilostane significantly reduced or suppressed the response of many genes to Fsh (like *wisp1*, testis *gapdhs*, *cldn11*, *inha*, *vt1* or *dmrt1*) showing that, in fish, important aspects of Fsh action follow indirect pathways and require the production of Δ4-steroids. What is more, most of the genes regulated by Fsh through steroid mediation were similarly regulated by Lh (and/or androgens). In contrast, the response to Fsh of other genes was not suppressed in the presence of trilostane. These latter included genes encoding for anti-mullerian hormone, midkine a (pleiotrophin related), angiopoietine-related protein, cyclins E1 and G1, hepatocyte growth factor activator, insulin-like growth factor 1b/3. A majority of those genes were preferentially regulated by Fsh, when compared to Lh, suggesting that specific regulatory effects of Fsh did not depend on steroid production. Finally, antagonistic effects between Fsh and steroids were found, in particular for genes encoding key factors of steroidogenesis (*star*, *hsd3b1*, *cyp11b2-2*) or for genes of the Igf system (*igf1b/3*). Our study provides the first clear evidence that, in fish, Fsh exerts Δ4-steroid-independent regulatory functions on many genes which are highly relevant for the onset of spermatogenesis.

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Introduction

In vertebrates, reproductive function is under the control of multiple factors acting in a cascade of regulations known as the brain-pituitary-gonadal (BPG) axis.

The male gonad is separated into two compartments, each with specific functions: the tubular compartment where spermatogenesis takes place to produce spermatozoa and the interstitial compartment which produces most of the steroids. Spermatogenesis is under the dual control of gonadotropins and sexual steroids. In mammals, each of the two gonadotropins, FSH and LH, have well targeted actions on the testis due to the exclusive presence of FSH receptors in the seminiferous epithelium, mostly on Sertoli cells and of LH receptors in the interstitial tissue. Despite rare reports on FSH receptors being detected in the germ cell line [1], the general consensus is that germ cell development is supported indirectly by gonadotropins. FSH stimulates the Sertoli cells to produce extracellular matrix proteins, growth factors and cytokines which in turn regulate germ cell proliferation and differentiation. LH stimulates the production of androgens by the Leydig cells. Androgens are required for normal development of spermatogenesis as illustrated by the impaired spermatogenesis in mutant mice lacking the nuclear androgen receptor either specifically in Sertoli cells (SCARKO) or in all testicular cell types expressing the nuclear AR (ARKO) [2,3].

This scheme is probably more complex since, at least in rodents, FSH action may involve or require the steroid pathway. In *hpg* mice that are devoid of gonadotropin production, FSH supplementation has been shown to induce Leydig cell function, probably indirectly through Sertoli cell stimulation [4,5]. Moreover, the development of round spermatids induced by FSH requires androgen action since the FSH effect is suppressed in *hpg*.SCARKO or *hpg*.ARKO mice [6].

The situation seems quite different in teleost fish, since in these species it has been established that both gonadotropins efficiently up-regulate steroidogenesis in testis and ovary, although some gonadal maturation stages can be more sensitive to one or the other gonadotropin [7–9]. This stimulating effect of Fsh and Lh is achieved through the up-regulation of genes encoding key players of steroid synthesis [10–12]. This particular feature can be explained by the expression of Lh receptors and Fsh receptors in the same interstitial cell type - probably Leydig cells - a situation described in an increasing number of primitive or evolutionarily advanced teleostean fish species like eel [13], African catfish [14], zebrafish [12], honeycomb grouper [15] and Senegalese sole [16]. In rainbow trout, this scheme ought to explain the potency of Fsh

to stimulate steroidogenesis since we showed that Fsh was not able to activate the Lh receptor efficiently [17].

Both Fsh and androgens are involved in the onset of spermatogenesis in fish. In Japanese eel, recombinant eel Fsh or 11-ketotestosterone (11KT) induced all steps of spermatogenesis from immature testicular explants cultured *in vitro* [18]. Moreover, the Fsh-induced spermatogenesis was inhibited by trilostane. The authors concluded that in this species the main role of Fsh in spermatogenesis would be to induce the production of 11KT, which in turn would regulate Sertoli cell function and, indirectly, germ cell proliferation/differentiation [13]. However in sea bass, Fsh but not Lh, was able to trigger spermatogenesis *in vivo* [19]. In salmonids, Fsh is the only circulating gonadotropin during the early stages of the reproductive cycle when A-spermatogonia actively proliferate and commit into differentiation [20]. Furthermore, Fsh induces the proliferation of spermatogonia in mixed cultures of trout somatic and germ cells [21]. In contrast, testosterone or 11KT had no effect on spermatogonia proliferation [21]. Thus, part of Fsh action is likely mediated by steroids but these latter may not fulfill all Fsh functions required to initiate spermatogenesis. In teleosts little is known about Fsh actions that would be independent of steroids. We recently demonstrated that *in vitro* Fsh treatment greatly modified the testicular transcriptome in trout and that this effect significantly differs from the effect of Lh [10]. The present work aimed at distinguishing the steroid independent actions of Fsh on testicular gene expression from those mediated by the steroids. Using a large scale transcriptomic analysis, we clearly demonstrate that Fsh acts both independently of and through the Δ4-steroid production. In addition, our data suggest that the specific regulatory effects of Fsh (in comparison to Lh), were mainly independent of steroid production. Finally, we find that Fsh and steroids may also have antagonistic regulatory effects, underlining the complex coordinated regulation of spermatogenesis by the different reproductive hormones.

Materials and Methods

Animals and *in vitro* organotypic culture

Ethics statement. Animals were bred and treated according to the guidelines for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. This project was approved by the local animal care and ethics committee of INRA under agreement n° B0009. The personnel were trained and qualified for animal experimentations.

Procedure. An all-male population of rainbow trout (*Oncorhynchus mykiss*) was obtained from the INRA experimental fish farm (PEIMA, Drennec, France) and kept in the laboratory facilities at 12°C under natural photoperiod until experimentation. Fish were deeply anesthetized in 1‰ 2-phenoxyethanol then killed by a blow to the head. Testes were removed, weighed and kept on ice in synthetic L15 media as modified by Loir [22] until preparation for culture. According to the macroscopic aspect of the testes and to the calculated gonadosomatic index (GSI), only fish with GSI≤0.15% were kept. Gonads were collected and cut into 1 mm^3 pieces using an automatic tissue chopper. All explants were pooled and mixed and about 10 testis fragments were randomly distributed (60–80 mg per well) onto Nunc polycarbonate membrane inserts in 24-well plates filled with 300 µL of culture medium supplemented with 2% Ultroser SF. Six replicate wells were used for each treatment. Incubation was performed for 96 h, at 12°C. Medium and hormones were replaced after 48 h of incubation. At the end of the incubation, tissues and culture media were centrifuged for 10 min at 200*g*. Tissues were frozen at −80°C in 1.2 mL of TRIzol® until RNA extraction. Culture media were frozen at −20°C until steroid radioimmunoassay.

Experiment 1 was carried out to evaluate the transcriptomic action of Fsh in the presence of trilostane, an inhibitor of the 3 beta-hydroxysteroid dehydrogenase. The pool of testis explants was issued from 58 fish (GSI mean = 0.089±0.037%). Explants were incubated in the absence or the presence of purified pituitary salmonid Fsh (500 ng/mL) alone or in combination with 10 µg/mL trilostane ($C_{20}H_{27}NO_3$, CHEMOS GmbH, Germany). A pre-incubation with trilostane was carried out for 1 hour before adding Fsh. These samples were used for the large scale transcriptomic analysis on trout cDNA nylon membrane arrays.

The same conditions were used in Experiment 2 to evaluate the action of Fsh and androgens on the transcription of a few candidate genes selected from Experiment 1. The pool of testis explants originated from 26 fish (GSI mean = 0.104±0.028%). Explants were incubated in the absence or the presence of purified Fsh (500 ng/mL) and of 2 biologically active androgens: 11-ketotestosterone (11KT) and 17α-methyl testosterone (MT, 17α-methyl-4-androsten-3-one) at the concentration of 300 ng/mL (about 10^{-6} M). This concentration was close to the 11KT concentration measured in the culture medium after 48 h of incubation with Fsh in Experiment 1.

A few pieces of testis tissue were also fixed on the day of sampling in Bouin's solution for histological examination. Fixed gonads were dehydrated and embedded in paraffin, and 5 µm sections were cut and stained with Regaud-Haematoxylin-Orange G-Aniline blue. The maturity stage of the gonads was evaluated based on the presence and on the relative abundance of the most developed germ cells, according to a classification described previously [20]. In each well, about 50% of explants were in stage I–II of testis maturation. These two gonadal stages are characterized by the presence of a large majority of A-spermatogonia. The remaining explants corresponded to Stage III which is characterized by a large number of B-spermatogonia and the appearance of meiotic cells (spermatocytes and rare spermatids).

Steroid measurement

To denature steroid binding proteins which may interfere with the steroid antibody, media were heated at 60°C for 20 min and centrifuged at 3000*g*, at 4°C for 15 min. Levels of 11KT were measured by specific radioimmunoassay (RIA) in culture media from Experiment 1 according to Fostier et al. [23]. Each sample was assayed in duplicate. The assay sensitivity was 80 pg/mL and the cross reactivity with testosterone or adrenosterone was 10%. The inter- and intra-assay coefficients of variation were 15% and 6%, respectively.

cDNA nylon membrane array experiment

RNA extraction and cDNA target synthesis. Total RNA was extracted using TRIzol® reagent and further purified with the NucleoSpin® RNA II kit (Macherey Nagel). RNA concentrations were quantified using the NanoDrop ND-1000 (Thermo Scientific) and RNA quality was determined using the Bioanalyser 2100 (Agilent). For cDNA target labeling, 5 µg total RNA were reverse-transcribed for 2 h at 42°C in the presence of radiolabelled dNTP (30 µCi [alpha-^{33}P] dCTP, 120 µMdCTP, 20 mM each dATP, dTTP, dGTP) using an oligo(dT) primer and 400 units of Superscript II reverse transcriptase (Invitrogen). RNA was degraded at 68°C for 30 min with 1 µL 10% SDS, 1 µL 0.5 M EDTA and 3 µL 3 M NaOH. The reaction was then equilibrated at room temperature for 15 minutes and neutralized by the addition of 10 µL 1 M Tris-HCl and 3 µL 2 N HCl.

Nylon membrane array hybridization and raw data production. cDNA arrays were generated by CRB GADIE (http://crb-gadie.inra.fr/) as previously described [24]. Prehybridization of the membranes was performed at 65°C for 4 h in 5X Denhardt's, 5X SSC and 0.5% SDS. Labelled cDNA targets were denatured at 95°C for 5 min and incubated with microarrays for 48 h at 65°C in the same buffer. The membranes were then washed three times for 1 h at 68°C in 0.1X SSC containing 0.2% SDS prior to a 48 hour exposure to phosphor-imaging plates. Plates were scanned using a FUJI BAS 500 and the BZscan software was used for signals acquisition [25]. Each membrane was also hybridized with a ^{33}P-labelled oligonucleotide (5′-TAATACGACTCACTATAGGG-3′) that recognizes the vector part of every PCR product to quantify the amount of spotted cDNA.

Normalization procedure. Expression data were normalized as previously described [26]. Briefly, raw data were corrected for the amount of spotted cDNA by dividing the sample signal (Si) of each spot by the corresponding vector signal (Vi). To avoid the bias affecting relative gene expression levels, the corrected signal of each spot was further multiplied by the median vector signal of all arrays for this same spot ((Si/Vi)×medVi). Expression values were then log2-transformed and submitted to a quantile-quantile normalization using the AMEN software (http://sourceforge.net/projects/amen/) [27]. Raw data as well as a normalized expression file are available at the GeneOmnibus public data repository http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46458.

Statistical and cluster analyses. Non-informative clones for which too small an amount of cDNA was spotted (oligonucleotide signal <3 times the background level in more than 20% of samples) were removed from the analysis. Clones were further filtered on their expression level (mean expression level ≥ to the median expression of all the experiment, at least in one experimental group). Finally, clones were filtered on a fold change ≥1.5 between control and Fsh-treated groups either in the absence or in the presence of trilostane.

Gonadotropin-responsive genes were then identified in the presence or not of trilostane, by comparing control groups to the Fsh-treated groups using the multi-class Limma statistical test with a false discovery rate (FDR) of 1% [28]. All differentially-expressed transcripts were then submitted to a hierarchical classification (Uncentered Pearson correlation measure).

Meta-analysis. Expression data obtained in a previous study [10] were used to investigate whether the 2 categories of Fsh-responsive genes (sensitive or insensitive to trilostane), were regulated by Lh. Samples in this dataset included explants of testis in early stages of spermatogenesis which were incubated for 4 days in the absence or in the presence of either Fsh or Lh (500 ng/mL). The data were normalized to avoid bias due to the different batches of nylon membranes as well as the 2 separate hybridization experiments. For each row (i.e. each gene) the expression signals of all arrays in one experiment were median-centered then normalized by the median expression of the second experiment. Only the clones demonstrating a statistically significant response to Fsh in both experiments were considered and submitted to a non-supervised hierarchical classification.

Real-time quantitative PCR (qPCR) experiments

The qPCR technique was used either to confirm changes in expression for selected transcripts identified from the microarray analysis or to examine additional transcripts previously found as being differentially regulated by gonadotropins and/or of putative interest regarding testis functions (the list is given in Table 1). Two micrograms of total RNA were submitted to reverse-transcription (RT) using 1 µg random hexamers and 200 units of MMLV reverse transcriptase (Promega) for 75 min at 37°C in a final of volume of 25 µL. Real-time PCR assays were performed on the StepOne^{TM} Real-Time PCR System (Applied Biosystems) using 4 µL of 1:30 diluted RT products, 1 µL of mixed oligo primers (0.6 µM for both reverse and forward primers) and 5 µL of Fast SYBR® Green Master Mix (Applied Biosystems). The amplification program consisted of an initial denaturation at 95°C for 20 sec; 40 cycles of 95°C for 3 sec, then 60°C for 30 sec. A final progressive increase of temperature (0.5°C/sec) has been carried out from 65 to 90°C at the end of the amplification for melting curve analysis.

Cycle threshold (Ct) was automatically setup and relative expression levels were normalized using a reference gene, *rps15* (clone 1RT58B15_B_A08). This gene was chosen on the basis of its invariant expression in spermatogenesis microarray experiments [26]. Its expression level also enabled its measurement at the same RT template dilution as selected candidate genes. All RT samples were measured in duplicates. Statistical analyses were performed with the Statistica software environment using the non-parametric ANOVA of Kruskal-Wallis and the Mann & Whitney's U test if a statistical difference ($p<0.05$) was observed between groups in the ANOVA analysis.

Real-time PCR oligonucleotide primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/) and were verified with the oligoanalyser 3.1 web interface (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) to avoid self- and hetero-dimer formation as well as hairpin structures. Nucleotide sequences of the primers were also systematically matched (BLAST algorithm) against the SIGENAE trout contig collection (som.10 version) to avoid non-specific annealing to other transcripts. PCR amplification effectiveness was verified using serial dilutions of pooled RT products. All primer sequences are provided in Table S1.

Results

To address the issue of steroid-mediated action of Fsh on testicular transcriptome, we carried out *in vitro* culture of testis explants incubated in the presence of Fsh alone or in combination with trilostane (Fsh+Tri), a known inhibitor of the 3 beta-hydroxysteroid dehydrogenase.

Trilostane efficiently suppressed basal and Fsh-stimulated androgen production

To determine the effectiveness of trilostane (Tri) in inhibiting Δ4-steroid synthesis throughout the culture period, we measured 11KT levels in the culture media. As expected, Fsh alone induced a strong stimulation of the production of 11KT (about 5 fold) over the culture period (Fig. 1). After the first 48 hours of incubation, the presence of trilostane resulted in a reduction of both the basal and the Fsh-stimulated 11KT production (76% and 86% decrease, respectively). Over the following 48 hour period, the basal production of 11KT was drastically reduced so that the trilostane effect was no longer observed. The Fsh-stimulated production was maintained under control condition but was nearly suppressed (−93%) in trilostane treated explants. Consequently, testis tissues incubated in the presence of both Fsh and trilostane were exposed to much lower levels of androgens than testis tissues incubated in the presence of Fsh alone (9.4±1.4 versus 134.00±15.10 ng/mL at the end of the culture).

Table 1. Additional candidate genes studied using qPCR.

SwissProt/GeneBank accession number	Gene Symbol	Annotation/Description	Preferential gonadotropin response	Steroid mediation	Predicted cellular origin
-	*igf1b/igf3*	Insulin-like growth factor 1b	Up Fsh	No, antagonism	Somatic
Q3HWG4	*igfbp6*	Insulin-like growth factor binding protein 6	Up Fsh	Partially	-
Q9I8S6	*cyp11b2-2*	Cytochrome P450 11 beta 2	Up Fsh, Lh	No, antagonism	-
Q71MM8	*fshr*	Follicle-stimulating hormone receptor	Up Fsh, Lh	Yes	Somatic
NM_001165391	*ccnd1*	G1/S-specific cyclin-D1	Down Fsh	No	-
Q71MM9	*lhcgr*	Luteinizing hormone receptor	Up Fsh, Lh	Yes	Somatic
O95633	*fstl3*	Follistatin-related protein 3 precursor	Up Fsh	No, antagonism	
NP_001117674	*star*	Steroidogenic acute regulatory protein, mitochondrial precursor	Up Fsh, Lh	No, antagonism	Somatic

These genes were of interest since they were previously found to be differentially regulated by gonadotropins (Sambroni et al., 2013).
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Trilostane modified the Fsh regulation of testicular gene expression

The variations of the transcriptome were analyzed at a large scale using trout cDNA microarrays. The effects of Fsh on testicular gene expression were studied after 4 days of incubation because we previously showed that Fsh and Lh modified testicular transcriptome more efficiently after a 4-day treatment compared to shorter durations. The microarray data were analyzed with AMEN software. After a double filtration on expression level and fold change, followed by a Limma analysis (FDR 1%; see M&M), 102 clones corresponding to 96 non redundant (NR) genes were found significantly differentially expressed between control and Fsh-treated conditions or between trilostane and Fsh+trilostane groups. All the information on annotation together with response to Fsh, trilostane and Lh for these 102 clones is provided in the searchable file S1. (Note that the responsiveness to Lh was determined in a previous study [10]). The hierarchical classification of the genes allowed the segregation of 5 main clusters of transcripts with correlated variations along the samples (Fig. 2).

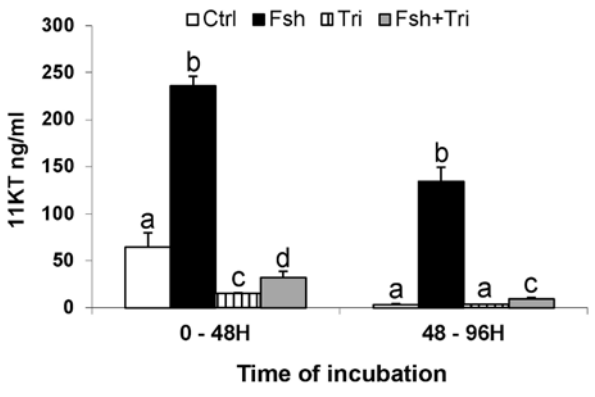

Figure 1. Evaluation of trilostane treatment efficiency. 11KT production in culture media after 48 h and from 48 to 96 h of incubation in the absence or in the presence of trout purified Fsh (500 ng/mL) alone or in combination with trilostane (10 µg/mL). Culture media were replaced after 48 h. Each bar represents the mean ± SD of 6 replicates. Different letters for each incubation duration indicate that treatments have significantly different effects as determined by non-parametric Mann & Whitney test ($p<0.01$).
doi:10.1371/journal.pone.0076684.g001

Overall, the great majority (74 out of 102) of the differentially-expressed transcripts were found to be up-regulated by Fsh.

Fsh action mediated by steroid production. In Cluster 1, 39 genes were up-regulated by Fsh and this up-regulation was greatly inhibited in the presence of trilostane (Fig. 3). This implies that the responsiveness of these genes to Fsh requires the production of steroids. Cluster 1 could be subdivided in 2 groups of genes: those for which the response to Fsh was suppressed by trilostane (Fig. 2, Cluster 1a) as for *nr2f2*, *gapdhs*, *slc26a4*, *smtn*, *ndpkz3*, *canx*, *fblim1*, *dmd* or *cebpd1* and those for which the response to Fsh was strongly reduced but not totally inhibited (Fig. 2, Cluster 1b) as for *dmrt1*, *cldn11*, *etnk1*, *cth*, *plg*, *sptbn1*, *vt1*, *timp2* and *mmp19* (Table 2). For some representative genes of Cluster 1, we confirmed by qPCR that the presence of trilostane led to a complete or drastic loss of the response to Fsh, supporting the hypothesis that Fsh indirectly regulated their expression through the production of steroids (Fig. 4). The up-regulation of three additional transcripts *fshr*, *lhcgr* and *igfbp6* was also found to be mediated by steroids.

Additional evidence of steroid-dependent Fsh action was obtained in Experiment 2 where we analyzed the effects of 11KT and MT on the expression of candidate genes and compared in the same experimental design the effect of Fsh. As expected, Fsh and androgens did up-regulate the steady-state levels of two transcripts that were sensitive to trilostane, *slc26a4* and *inha* (Fig. 5A). However in our experimental conditions androgens were not able to stimulate *vt1*, *wisp1* and *mmp19*.

Fsh action independent of steroid production. Most interestingly Clusters 2 and 5 pointed out Fsh up-regulations or down-regulations, respectively, that were both maintained in presence of trilostane (Fig. 3). This demonstrates that Fsh could act on gene expression independently of the mediation of Δ4-steroids. Among the genes of interest in these groups (Table 3), we notice genes related to cholesterol biosynthesis (*hmgcr*, *abca1*), lipid metabolism (*fasn*) or steroidogenesis (*hsd3b1*). Other genes were encoding for neurohormones (*tac1*), growth factors (*mdka*, *amh*) and proteins involved in the cell cycle (*ccne1*, *ccng1*, *ing4* and *mcm7*) or in cell shape and cytoskeleton (*ezr*, *des*).

For selected genes of that category (Fig. 6), the qPCR measurement provided compelling evidence that Fsh action was independent of Δ4-steroid production. The regulation by Fsh either was not inhibited by trilostane (Fig. 6A: *mdka*, *amh* and *ccnd1*), or was even amplified in the presence of trilostane (see below and Fig. 6B).

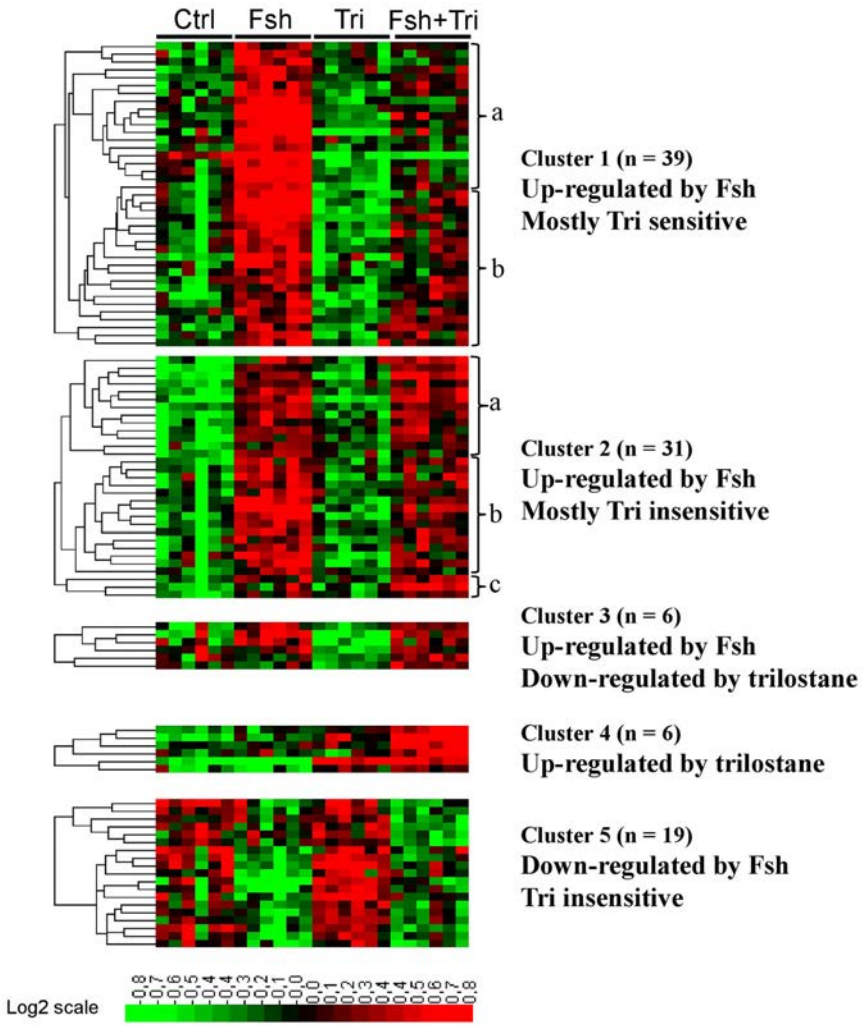

Figure 2. Expression of Fsh-responsive genes. Heatmap representation of the hierarchical classification of the 102 clones differentially regulated in trout testis after an *in vitro* 4-day incubation without any substance (Ctrl) or with Fsh alone at 500 ng/mL (Fsh), trilostane alone at 10 μg/mL (Tri) or with both Fsh and trilostane (Fsh+Tri). Trilostane was added 1 h before Fsh addition in the medium. Media and hormones were renewed after 2 days. Clones segregated into 5 main groups, corresponding to genes which were up- or down-regulated by Fsh and sensitive or not to trilostane. Normalized expression values are shown according to the scale bar. Each line represents a clone and each column is a sample.
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Fsh and steroids show cooperative or antagonistic effects on gene expression. Cluster 3 regrouped genes for which basal expression was decreased by trilostane alone, suggesting a stimulatory action of Δ4-steroids, whereas the Fsh-stimulated expression level was not modified by trilostane. This indicates that Fsh and steroids regulate similarly those genes.

Cluster 4 was not expected because it grouped few genes for which i) Fsh alone had no significant modulatory effect, ii) trilostane stimulated basal gene expression, possibly revealing an inhibitory action of Δ4-steroids and iii) a stimulatory effect of Fsh was observed only in the presence of trilostane. This indicates that high levels of steroids induced by Fsh could prevent or mask Fsh action on gene expression. In coherence with the microarray data, qPCR measurements confirmed a group of transcripts characterized by an amplified response to Fsh in the presence of trilostane (Fig. 6B: *fstl3*, *hsd3b1*, *cyp11b2-2* and *igf1b*).

Direct evidence of the antagonistic effects between Fsh and steroids was demonstrated by comparing the impact of androgens and Fsh *in vitro* on candidate genes: while up-regulation by Fsh was confirmed, androgen treatment significantly down-regulated *igf1b*, *star*, *hsd3b1* and *cyp11b2-2* (Fig. 5B).

Specific effects of Fsh are not mediated through Δ4-steroid

In a previous study we showed that Fsh and Lh had both common and distinct effects on testicular transcriptome [10]. In the present study, we addressed the question of whether sex steroids mediated the common actions of Fsh and Lh, while the steroid-independent response to Fsh would correspond to genes specifically regulated by Fsh and not by Lh. To proceed to the meta-analysis, we combined data obtained in the present study with those reported previously [10] : after a step of normalization between the two experiments, we performed an unsupervised hierarchical classification including 44 arrays and the 58 clones found to be regulated by Fsh in the two studies (Fig. 7). Remarkably, we observed that the genes up-regulated by Fsh and sensitive to trilostane were also up-regulated by Lh (Cluster A). Conversely, the genes up-regulated by Fsh but insensitive to trilostane were not responsive to Lh (Cluster B).

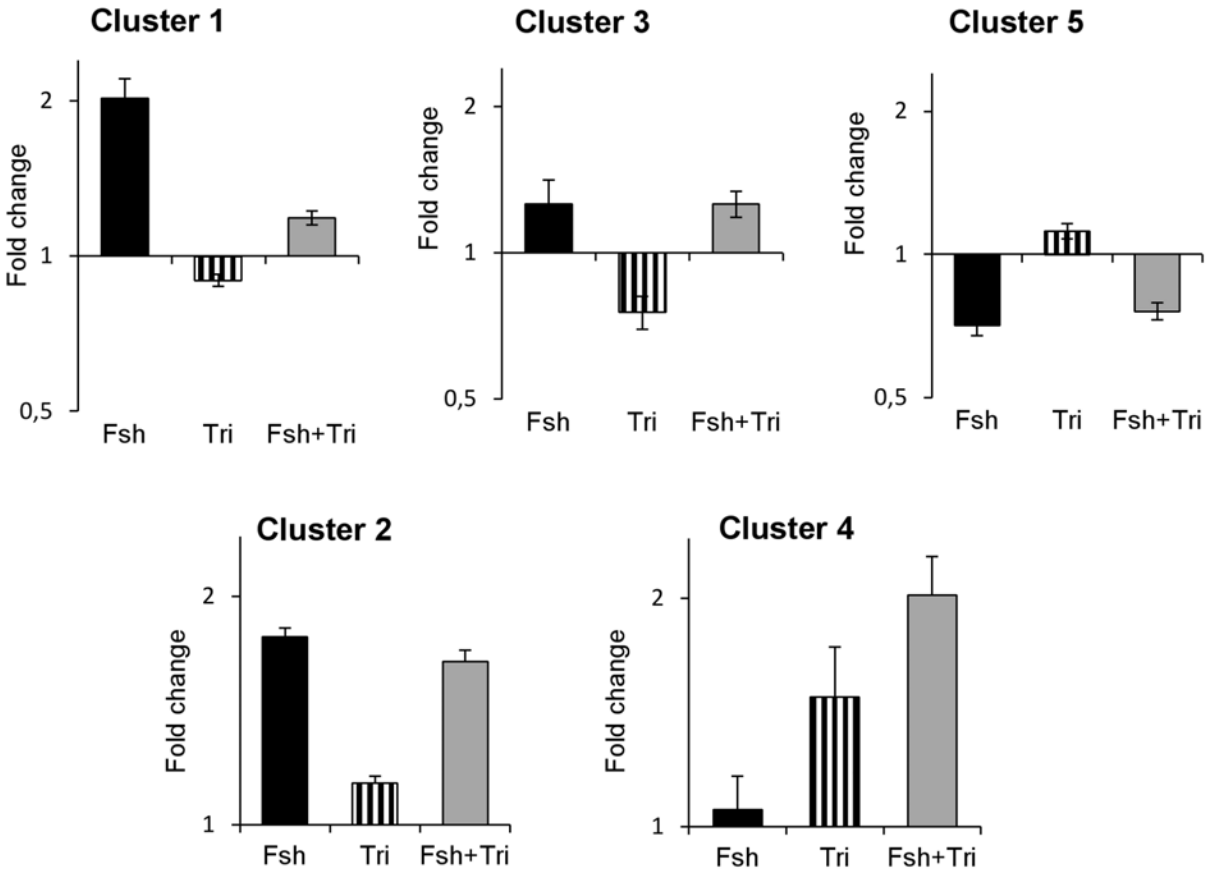

Figure 3. Representation of the mean fold-change in each cluster. Fold changes are relative to the control group. All the 102 clones included were individually found significantly affected by Fsh. Bars represent mean ± SEM. Y-axis is log-2 scaled and value 1 represents the control level. For convenience, note that the clusters are not shown in numerically ascending order.
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Discussion

Since both Fsh and Lh efficiently stimulate steroidogenesis in fish, deciphering their respective roles along the reproductive cycle remains a crucial question. Furthermore, whether Fsh acts directly on the spermatogenic compartment or through steroid production by Leydig cells remains unclear in many teleostean species studied so far. In this context, we addressed the question of whether the regulatory effects of Fsh on gene expression could be mediated independently of the production of biologically active steroids. Our conclusions are based on a large scale transcriptomic analysis.

To distinguish steroid-independent regulatory effects of Fsh from those mediated through the steroids, we used the trilostane, a known inhibitor of the 3β-hydroxysteroid dehydrogenase/D5-D4 isomerase (3β-HSD). Trilostane blocks the production of Δ4-steroids that include testosterone, 11 ketotestosterone, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), and estradiol which are considered as the main biologically active sexual steroids in fish [29,30]. As expected, we showed that the dose of trilostane used in the present study was effective in inhibiting the release of 11KT in the culture media, indicating that it inhibited the upstream processes in Δ4-steroid synthesis effectively. However, we cannot totally exclude the fact that the trilostane-insensitive actions of Fsh could in fact be mediated through the production of delta5 steroids, like dehydroepiandrosterone. Nevertheless, the delta 5 steroid pathway has been detected in immature trout ovaries [31], not in immature testis [32] and it is generally assumed that the biosynthesis of steroids in fish testis mainly follows the delta 4 pathway [33].

The analysis of the effects of trilostane on the changes induced by Fsh in the testicular transcriptome discloses two main mechanisms underlying the action of Fsh: the first mechanism involves the production of steroids, which in turn probably relay Fsh action. The second mechanism implies that Fsh acts independently of the Δ4-steroid mediation.

Fsh action mediated by steroids

For specific transcripts mostly grouped in Cluster 1, the stimulatory effect of Fsh was totally or nearly suppressed when steroidogenesis was inhibited. This clearly indicates that Fsh acts indirectly on the corresponding genes through the production of sex steroids. This observation is in agreement with the steroidogenic activity of Fsh and the cellular expression of its cognate receptor on fish Leydig cells. Furthermore, several of these genes were also found to be up-regulated by androgen *in vivo* [34] or *in vitro* (this study). This supports the idea that androgens are the mediators of the effects of Fsh on those particular genes. Conversely, *in vitro* androgen treatment was unable to increase the steady-state level of mRNA of some genes (*vt1*, *wisp1* and *mmp19*) suggesting that the action of Fsh could be mediated by steroids other than 11KT and MT. Among the genes up-regulated through the mediation of steroids, we found the gonadotropin receptor transcripts, *fshr* and *lhcgr*. Although we cannot incriminate a particular steroid in the present study, our data are consistent

Table 2. Representative transcripts up-regulated by Fsh and sensitive to trilostane treatment.

Gene Symbol	Gene description	Lh regulation[1]	Testicular expression profile[2]	*In vivo* androgen response[3]
Fsh-response abolished by trilostane treatment				
-	14 kDa apolipoprotein	Yes	Somatic	-
cebpd1	CCAAT/enhancer-binding protein delta (C/EBP delta)	Yes	Somatic	-
nr2f2	COUP transcription factor 2 (COUP-TF2)	No	-	-
canx	Calnexin precursor	-	Somatic Up stage 8	-
-	Cytokine-like nuclear factor n-pac	-	-	-
cyp46a1	Cytochrome P450 46A1	No	Somatic	Up at day 7
dmd	Dystrophin	Yes	Meiotic Up stage 8	-
fdps	Farnesyl pyrophosphate synthetase (FPP synthetase)	-	-	-
fblim1	Filamin-binding LIM protein 1 (FBLP-1)	-	-	-
gapdhs	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	-	-	-
it	Isotocin-I	Yes	Somatic Up stage 8	-
-	Similar to vertebrate acyl-CoA thioesterase 11	-	A-spermatogonia	-
slc26a4	Similar to vertebrate solute carrier family 26	Yes	Somatic Up stage 8	Up at day 7
ndkpz3	Nucleoside diphosphate kinase-Z3	Yes		-
-	Putative uncharacterized protein (Fragment)	Yes	Somatic Up stage 8	-
smtn	Smoothelin-b (Fragment)	No	Somatic	Up at day 14
-	Similar to vertebrate protein tyrosine phosphatase, receptor type, F	Yes	-	-
tpd52l1	Tumor protein D53 homolog	Yes	-	-
wisp1	WNT1-inducible-signaling pathway protein 1 precursor	No	Somatic Up stage 8	-
Fsh-response significantly reduced by trilostane treatment				
ctl1	Cathepsin L	Yes	Somatic	-
cebpd2	CCAAT/enhancer binding protein delta2	Yes	Somatic	-
lrrc8	Leucine-rich repeat-containing protein 8C	Yes	-	-
cldn11	Claudin 11a	-	Somatic Up stage 8	Up at day 7
cth	Cystathionase (cystathionine gamma-lyase)	Yes	Somatic Up stage 8	Up at day 7
cyp2m1	Cytochrome P450 2M1	Yes	Somatic	Up at day 7
dmrt1	Doublesex- and mab-3-related transcription factor 1	Yes	Somatic	Up at day 7
etnk1	Ethanolamine kinase 1	Yes	Somatic Up stage 8	-
-	Homolog of Homo sapiens Transport-secretion protein 2.2	No	Somatic Up stage 8	-
inha	Inhibin	Yes	Somatic	Down at day 7
mmp19	Matrix metalloproteinase-19 precursor	Yes	A-spermatogonia	-
plg	Plasminogen	Yes	Somatic Up stage 8	Up at day 7
cpvl	Probable serine carboxypeptidase CPVL precursor	Yes	-	-
-	Putative uncharacterized protein	No	Somatic	-
-	Putative uncharacterized protein (Fragment)	-	Somatic	Up at day 7
rbm47	RNA-binding protein (FLJ20273), transcript variant 1	-	-	-
slc9a3r1	Solute carrier family 9 (Sodium/hydrogen exchanger), isoform 3 regulatory factor	Yes	-	-
sptbn1	Spectrin beta chain, brain 1	Yes	Somatic	-
timp2	Tissue inhibitor of metalloproteinase 2	Yes	Somatic Up stage 8	Up at day 7
vt1	Vasotocin-neurophysin	Yes	Somatic Up stage 8	Up at day 7

The corresponding genes segregated in Clusters 1or 2 and the response to Fsh was further identified as highly or moderately sensitive to trilostane in pairwise comparisons (Limma statistical test, p≤5%). When the information was available, we indicated the response to Lh ([1]) Sambroni et al., 2013), the testicular expression profile as well as the *in vivo* regulation by androgens ([2])Rolland et al., 2009 and ([3]) Rolland et al., 2013). -: not determined.

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with the androgen-induced increase of the steady-state levels of *fshr* and *lhcgr* in African catfish testis [35]. This observation combined with high gonadotropin plasma levels measured at the end of the reproductive cycle [20] could explain the large increase of *fshr* and *lhcgr* transcript expression observed in the spawning trout [17]. This finding highlights an efficient amplification loop of the gonadotropin signaling pathways at that stage.

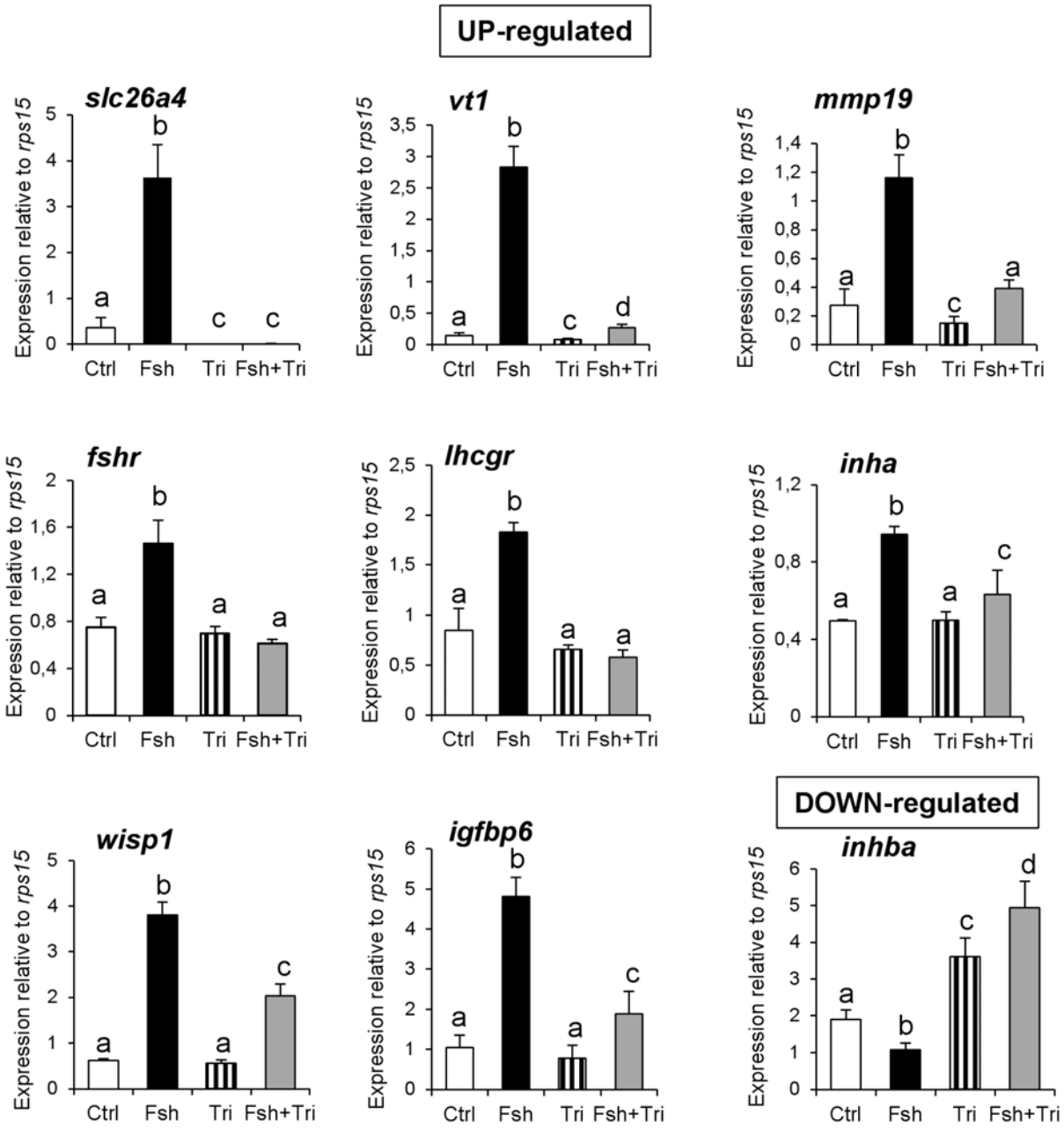

Figure 4. Steroid-mediated action of Fsh on the steady-state level of mRNA transcripts measured by qPCR. *slc26a4*, *vt1*, *mmp19* and *wisp1* and *inha* genes segregate in Cluster 1. The *inhba* gene belongs to Cluster 4. Three additional transcripts (*fshr*, *lhcgr* and *igfbp6*) previously demonstrated as up-regulated by gonadotropins are found to behave like genes in Cluster 1. Note that Fsh regulations of *inha*, *wisp1* and *igfbp6* are only partially lost in the presence of Tri. Bars represent mean ± SD of 5 to 6 replicates. Expression data were normalized to the reference gene *rps15*. Different letters indicate that treatments are significantly different as determined by non-parametric Mann & Whitney test.
doi:10.1371/journal.pone.0076684.g004

The mediation through steroid production was also established for a few genes which were negatively regulated by Fsh. Among those, *inhba* encodes for the beta A subunit of activin. This subunit is part of growth factors involved in testis physiology and was previously found strongly down-regulated by androgens [34]. Another transcript in this group, *sl*, encodes for the somatolactin hormone, a fish hormone mainly expressed in the pituitary and capable to stimulate testicular androgen production in the gonads [36].

We noticed that in several cases the response to Fsh was significantly reduced but not fully suppressed in the presence of trilostane. Because in our experimental conditions the Fsh-induced steroid production was not totally suppressed by trilostane either (Fig. 1), we hypothesize that those genes are sensitive to low concentrations of steroids. However we cannot exclude a redundant regulation by Fsh and steroids for some of them, as evoked previously in mice for a few Sertoli cell transcripts [37].

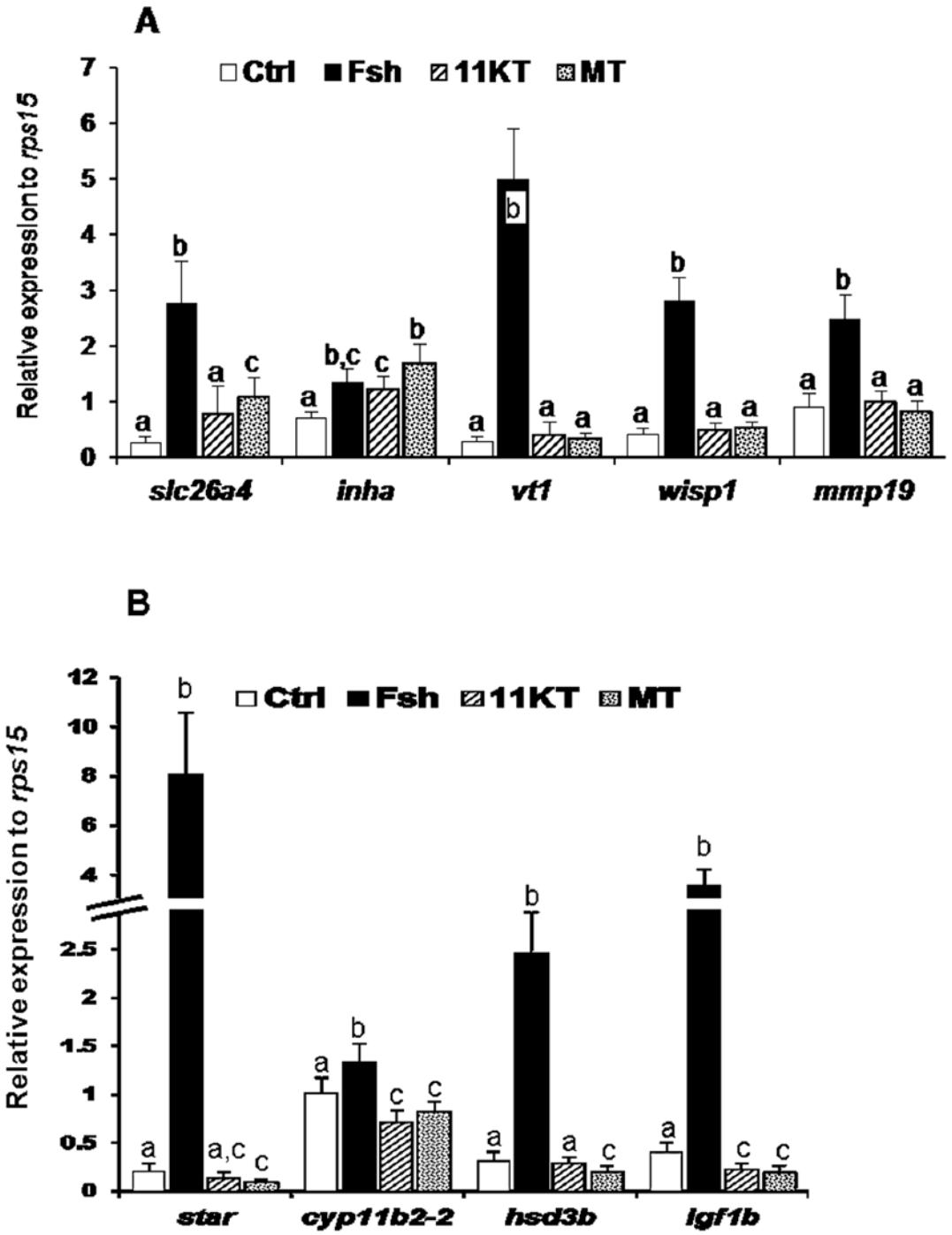

Figure 5. Real-time PCR measurement of candidate gene expression. Relative expression of selected mRNA transcripts in testicular explants cultured during 4 days in the absence (Ctrl) or presence of either Fsh (500 ng/mL), 11-ketotestosterone (11KT) or 17α-methyltestosterone (MT) at 300 ng/mL ($\sim 10^{-6}$ M). Bars represent mean ± SD of 5 to 6 replicates. Expression data were normalized to the reference gene *rps15*. Different letters indicate that treatments are significantly different as determined by non-parametric Mann & Whitney test.
doi:10.1371/journal.pone.0076684.g005

Since in fish both Lh and Fsh induce steroid production, we hypothesized that the genes regulated by Fsh through steroid mediation (Cluster 1) should also respond to Lh *in vitro*. Recently, we demonstrated that Fsh and Lh have common but also distinct effects on gene expression in rainbow trout testis [10]. Our meta-analysis disclosed that a majority of Cluster 1 genes was similarly regulated by Fsh and by Lh in our previous study, supporting the idea that for those genes, Fsh acts similarly to Lh mainly through the mediation of steroids.

To highlight the biological significance of the hormonal regulations described above, we performed additional data mining to retrieve the expression profiles of the candidate genes during trout testis maturation which were previously reported [26]. We found that genes for which the action of Fsh was mediated by

Table 3. Representative transcripts regulated by Fsh independently of Δ4-steroid production.

Gene Symbol	**Gene description**	**Lh regulation[1]**	**Testicular expression profile[2]**	***In vivo* androgen response[3]**
Genes up-regulated by Fsh				
hsd3b1	*3-HSD 1 protein*	*Yes*	*Somatic Down stage 8*	*Down*
hmgcr	3-hydroxy-3-methylglutaryl-coenzyme A reductase	-	-	-
aplp1	Amyloid-like protein 1 precursor	-	-	-
angptl7	Angiopoietin-related protein 7 Precursor	-	Gonia A	Down
abca1	ATP-binding cassette sub-family A member 1	No	Somatic	-
ctssb1	Cathepsin S	No	Somatic Down stage 8	-
ctss	Cathepsin S precursor	No	Somatic	-
slc7a3	Cationic amino acid transporter 3 (CAT-3)	No	-	-
ccng1	Cyclin-G1	-	-	-
eftud1	Elongation factor Tu GTP-binding domain-containing protein 1			
ezr	Ezrin (p81) (Cytovillin) (Villin-2)	-	-	-
fasn	Fatty acid synthase	-	Somatic Up stage 8	-
fra2	Fos-related antigen 2	No	Gonia B	-
hgfa	Hepatocyte growth factor activator	-	-	Down
hk1	Hexokinase-1	-	-	-
krt12	Keratin 12	No	Somatic Down stage 8	-
galns	N-acetylgalactosamine-6-sulfatase precursor	Yes	-	Up at day 7
odc1	Ornithine decarboxylase	No	Somatic	-
mdka	Midkine-related growth factor (Pleiotrophin related)	No	Somatic	-
tac1	Protachykinin 1 precursor	No	Somatic Up stage 8	-
-	Putative uncharacterized protein	No	Somatic	-
psmd1	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	No	-	Up at day 7
rbm47	RNA-binding protein (FLJ20273), transcript variant 1	-	-	-
rhobtb3	Rho-related BTB domain-containing protein 3	-	Somatic	-
slc25a4	Solute carrier family 25 member 43	Yes	Somatic	-
tfpi2	Tissue factor pathway inhibitor 2	No	Somatic Down stage 8	-
-	Toxin-1	Yes	-	-
Symbol	**Gene Description**	**Lh negative response**	**Testicular expression profile**	***In vivo* androgen response**
Genes down-regulated by Fsh				
rpl30	*60S ribosomal protein L30*	-	-	-
amh	Anti-mullerian hormone	No	Somatic Down stage 8	Down
des	Desmin	No	-	-
mcm7	DNA replication licensing factor MCM7	Yes	-	-
ccne1	G1/S-specific cyclin-E1	Yes	Somatic Down stage 8	-
cenpa	Histone H3-like centromeric protein A	Yes	Meiotic/post meiotic	-
onmydab	Homolog of Homo sapiens HLA class II histocompatibility antigen, DQB1*0602 beta chain	Yes	Somatic	-
ing4	Inhibitor of growth family, member 4	No	Somatic Down stage 8	-
onmy-uba	MHC class I heavy chain	Yes	Somatic	-
nfkb1	Nuclear factor NF-kappa-B p105 subunit	Yes	-	-
pol	Pol polyprotein (Fragment)	-	-	-
akt3	RAC-gamma serine/threonine-protein kinase	-	-	-
fcf1	rRNA-processing protein FCF1 homolog	-	-	-
Genes down-regulated by Fsh only in the presence of trilostane				
zfp658	*BC043301 protein (Fragment)*	-	-	-
purg	Purine-rich element-binding protein gamma	-	-	-
tmem106a	Tmem106a protein (Fragment)	-	-	Up at day 7

Up-regulated genes mostly segregated in Cluster 2 whereas down-regulated genes segregated in Cluster 5. The response to Fsh was further identified as insensitive to trilostane in pairwise comparisons (Limma statistical test, p≤5%). When the information was available, we indicated the response to Lh ([1]) Sambroni et al., 2013), the testicular expression profile as well as the *in vivo* regulation by androgens ([2])Rolland et al., 2009 and ([3]) Rolland et al., 2013). -: not determined.

doi:10.1371/journal.pone.0076684.t003

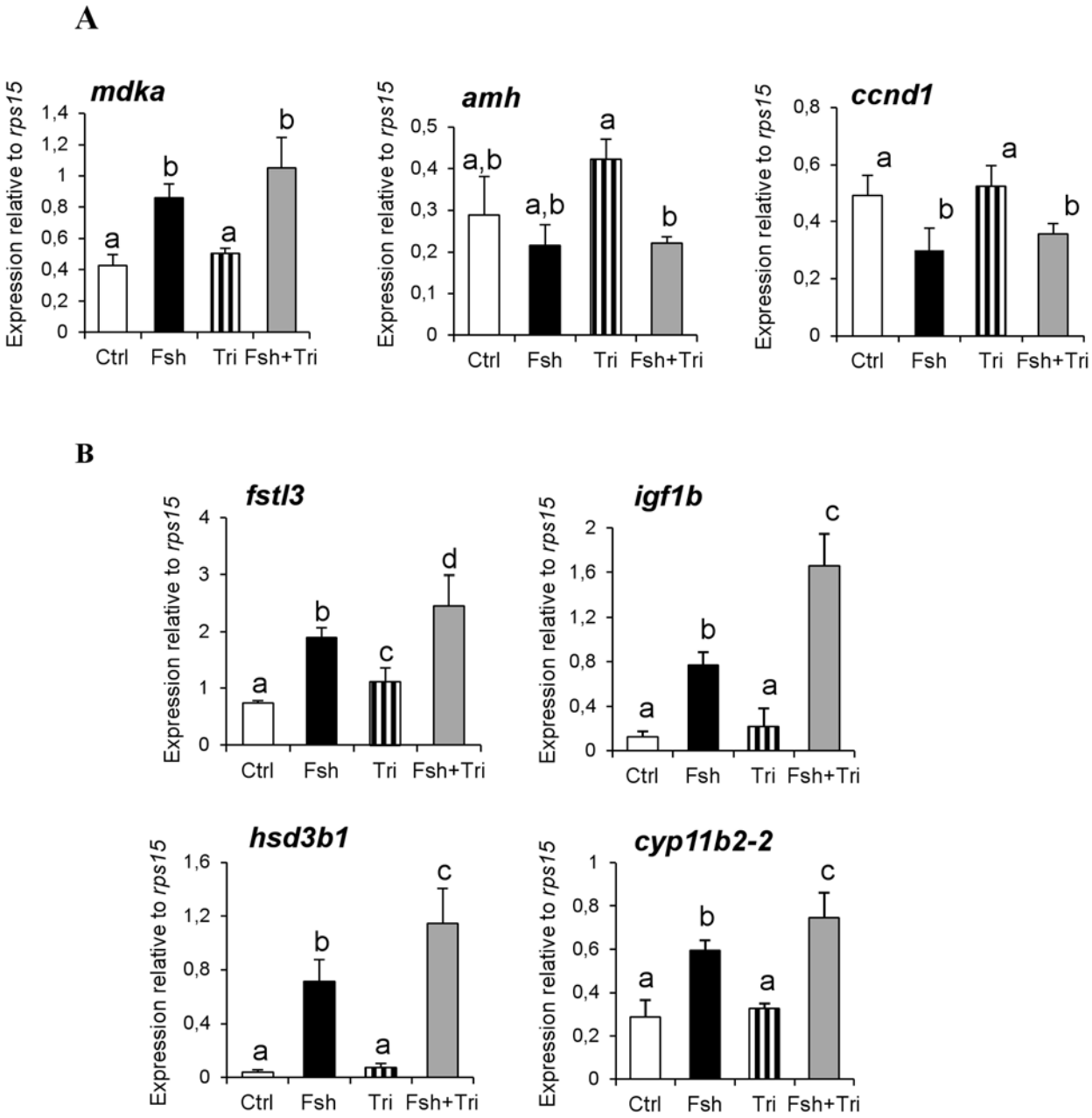

Figure 6. Steroid-independent action of Fsh on the steady-state level of mRNA transcripts measured by qPCR. The *mdka* and *hsd3b1* genes belong to Cluster 2 and the *amh* gene is segregated in Cluster 5. Four additional candidates previously demonstrated as being regulated by gonadotropins - *ccnd1*, *fstl3*, *igf1b/igf3* and *cyp11b2-2* - behave like genes of Cluster 2. Furthermore an increased response to Fsh in the presence of trilostane suggests an antagonism between Fsh and the Δ4- steroids. Bars represent mean ± SD of 5 to 6 replicates. Expression data were normalized to the reference gene *rps15*.
doi:10.1371/journal.pone.0076684.g006

steroids displayed a large increase or a decrease at the end of the reproductive cycle, when both gonadotropin hormones and steroids exhibit high plasma levels in salmonids (data not shown). Such a convergence between high levels of circulating hormones and maximal or minimal gene expression levels during the reproductive cycle reinforces the physiological consistency of our data.

Fsh action independent of steroids

Conversely, the effects of Fsh on genes grouped in Clusters 2 and 5 were maintained in the presence of trilostane, reflecting that a part of the Fsh action did not require the production of Δ4-steroid. Interestingly, in our meta-analysis, a majority of genes of this category (like *mdka*, *ctss*, *krt12* and *fosl2*) were also found preferentially up-regulated by Fsh when compared to Lh, reinforcing the idea of a steroid independent regulation. Several of these Fsh responsive genes including *amh*, *abca1*, *ezr*, *gapdhs*, *slc7a3*, *ccng1*, *cebpb*, and *fasn*, are known to be expressed in Sertoli cells in mice [38]. Furthermore, when comparing our data with those obtained in the rat after a 24 hour incubation of Sertoli cells with ovine FSH, we retrieve a number of genes (*ezr*, *hmgcr*, *odc1*, *fasn*, *des*) or pathways (Hgf system, amino acid transport, glycolysis)

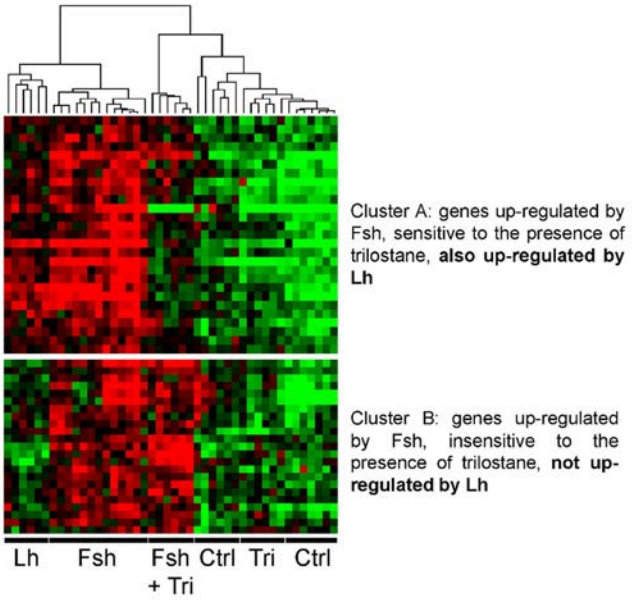

Figure 7. Meta-analysis: heatmap representation of the unsupervised hierarchical classification of the 58 clones found regulated by Fsh in the present study and in our previous study where Fsh and Lh effects on testicular transcriptome were measured (Sambroni et al., 2013).
doi:10.1371/journal.pone.0076684.g007

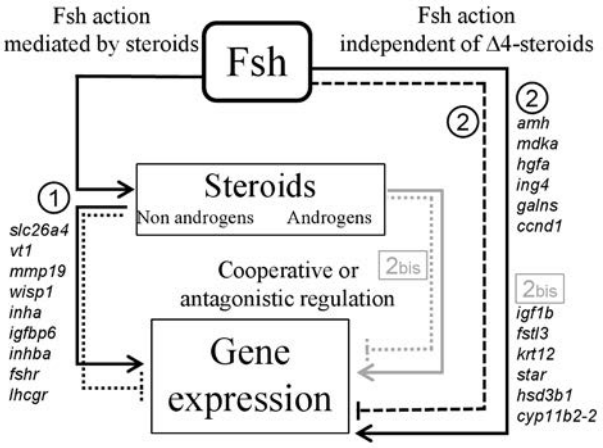

Figure 8. Summary of the mechanisms underlying Fsh action on gene expression in rainbow trout testis. 1- The primary action of Fsh is to stimulate steroidogenic cells to produce steroids which in turn regulate gene expression. 2- Fsh exerts specific regulatory effects independently of steroid mediation. 2 bis- In some cases steroids could have either an antagonistic or a redundant effect on gene expression. Plain lines with an arrow head indicate stimulatory effects whereas dotted lines illustrate inhibitory effects.
doi:10.1371/journal.pone.0076684.g008

that are responsive to Fsh in the two studies [39]. The similarities between the regulations observed in trout and rat suggest that specific functions of Fsh would directly target Sertoli cells and have been, at least in part, conserved throughout the evolutionary process.

Our data indicate that steroid-independent effects of Fsh occurs also in Leydig cells. We noted that genes involved in steroidogenesis are induced by Fsh and not by steroids. These genes include *hsd3b1* and *star*. Our data and the expression of the Fshr previously reported in fish on Leydig cells [12–14,16] suggest that Fsh could act directly on gene expression in those cells.

The *in vitro* Δ4-steroid independent action of Fsh on gene expression does not exclude the fact that steroids on their own could regulate the steady-state levels of some of these transcripts. In particular, genes encoding key factors involved in steroidogenesis were up-regulated by Fsh and down-regulated by androgens *in vivo*: *star*, *hsd3b1and cyp17a1* [34] or *in vitro*: *hsd3b1*, *star* and *cyp11b2-2* (this study). Such antagonistic regulatory effects suggest a short loop feedback by high concentrations of the sexual steroids that could be essential to allow a local fine-tuning of steroidogenesis. We also noted antagonistic effects between Fsh and steroids for genes involved in early germ cell proliferation and/or differentiation like *igf1b*.

Fsh-regulated factors and testicular functions

We gave particular attention to several transcripts regulated by Fsh independently of the mediation of steroids, as they encode growth factor related products or cytokines, that may be key factors in the mechanism of Fsh action on germ cell proliferation/differentiation or on Leydig cell functions. In this category, we can cite anti-mullerian hormone (*amh*), insulin-like growth factor 1b (*igf1b*), hepatocyte growth factor activator (*hgfa*), follistatin-like 3(*fstl3*) and midkine (pleiotrophin related) (*mdka*). Except for *igf1b*, they are also found expressed in the mouse gonad [40–42].

Amh is known to prevent spermatogonial proliferation and differentiation in fish [43,44]. Here, the down-regulation by Fsh of *amh* transcript was found to be insensitive to trilostane and therefore independent of Δ4-steroid production. This observation is consistent with the inability of androgens in regulating *in vitro amh* expression in trout (not shown) and in zebrafish [44] but in contradiction with 11KTdecreased amh mRNA levels in eel [43].

Igf1b (also named Igf3) is a new member of the Igf family which is preferentially expressed in teleost gonad [45]. Previous studies had demonstrated that Fsh and recombinant Igf1 stimulated spermatogonia proliferation in trout [21,46]. More recently, we found that Fsh stimulated the expression of the *igf1b* gene (but not *igf1a* or *igf2*) suggesting that this Igf form is a major mediator relaying the Fsh action on trout spermatogonia proliferation [10].A similar conclusion was reached in zebrafish (Schulz and col., unpublished data). In the present study, high levels of androgens decreased the level of *igf1b* transcript. (Note that estrogens and the progestin DHP also down-regulate the gonadal *igf1b* mRNA level in zebrafish and tilapia [47–49]). The negative influence of androgens on the *igf1b* transcript in rainbow trout may therefore limit the accumulation of spermatogonia during the mid and late testicular stages. In addition, we previously showed that administration of androgens tended to stimulate the expression of germ cell genes involved in meiotic differentiation [34]. Altogether, our data suggest a sequential cooperation between Fsh and sex steroids in trout: a primary function of Fsh in prepubertal males would be to stimulate the active accumulation of spermatogonia; in animals undergoing pubertal maturation, the increasing production of steroids would limit the proliferation of spermatogonia and favor their differentiation.

Hgf, which is activated upon endoproteolysis by Hgfa, was shown to block apoptosis and stimulate the proliferation of germ cells in the prepubertal rat testis [50]. Fstl-3 is a glycoprotein that binds and inhibits the action of TGFβ ligands such as activins, which have numerous developmental and regulatory activities within the gonads. The role of midkine/pleiotrophin family, secreted heparin-binding cytokines, in the regulation of testicular function, is unknown in fish and poorly documented in

vertebrates. However an interesting study reported an increase of germ cell apoptosis in mice having a dominant negative mutation of pleiotrophin [51]. Midkine increases activity of mitotic pathways in primordial germ cells in vitro, keeping them in a proliferative, less differentiated state [41]. Considering their functions, these 3 factors point to new strong candidate pathways that could specifically mediate Fsh regulation of germ cell survival or proliferation in fish.

In summary (Fig. 8), this study provides the first large scale evidence that Fsh controls gene expression in fish testis through two different mechanisms: the first one requires the production of steroids whereas the second mechanism requires a steroid-independent pathway. We point out new candidate pathways that could be involved in the primary effects of Fsh on early spermatogonial and/or Sertoli cell proliferation in trout, then on meiosis initiation, when Lh is not yet secreted. Finally, a few cooperative or antagonistic effects between Fsh and sex steroids are demonstrated. We anticipate that the knowledge gained from this study will provide new insights on the specific role of Fsh and on its cooperation with steroids in regulating fish spermatogenesis.

Supporting Information

Table S1 Sequences of primers used in qPCR experiments. The gene symbol, the accession number and the sequence of forward and reverse primers (5′-3′) used for qPCR measurements are indicated.
(DOCX)

File S1 Steroid-mediated and steroid–independent actions of Fsh on gene expression. The searchable excel file summarizes the effect of trilostane on the Fsh responsiveness of 102 testicular genes found to be regulated using the microarray approach (Clusters 1 to 5) and provides a comprehensive annotation including "Clone Name", "Gene Symbol", "Gene name", GeneOntology terms and IDs ("Biological process", "Molecular function" and "Cellular component"). Additional information, extracted from previous studies, is also reported and includes Lh responsiveness at stage I–II and stage III (Sambroni et al., 2013), testicular expression profile (Rolland et al., 2009) and androgen responsiveness (Rolland et al., 2013). The file also provides the quantile-quantile normalized expression data (Log-2 transformed) of the 102 clones found to be significantly regulated. (XLSX)

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Author Contributions

Conceived and designed the experiments: FLG ES JJL. Performed the experiments: ES FLG. Analyzed the data: ES FLG. Contributed reagents/materials/analysis tools: ES FLG. Wrote the paper: ES FLG JJL.

References

1. Baccetti B, Collodel G, Costantino-ceccarini E, Eshkol A, Gambera L et al. (1998) Localization of human follicle-stimulating hormone in the testis. The FASEB Journal 12: 1045–1054.
2. De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M et al. (2004) A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101: 1327–1332.
3. O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH (2009) Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: Data from mutant and genetically modified mice. Molecular and Cellular Endocrinology 306: 2–8.
4. O'Shaughnessy PJ, Bennett MK, Scott IS, Charlton HM (1992) Effects of FSH on Leydig cell morphology and function in the hypogonadal mouse. J Endocrinol 135: 517–525.
5. Baines H, Nwagwu MO, Hastie GR, Wiles RA, Mayhew TM et al. (2008) Effects of estradiol and FSH on maturation of the testis in the hypogonadal (hpg) mouse. Reprod Biol Endocrinol 6: 4.
6. O'Shaughnessy PJ, Monteiro A, Verhoeven G, De Gendt K, Abel MH (2010) Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors. Reproduction 139: 177–184.
7. Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H (1992) Properties of common carp gonadotropin I and gonadotropin II. General and Comparative Endocrinology 85: 217–229.
8. Suzuki K, Nagahama Y, Kawauchi H (1988) Steroidogenic activities of two distinct salmon gonadotropins. General and Comparative Endocrinology 71: 452–458.
9. Planas JV, Swanson P (1995) Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. Biol Reprod 52: 697–704.
10. Sambroni E, Rolland AD, Lareyre JJ, Le Gac F. (2013) Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis. J Mol Endocrinol 50: 1–18.
11. Kazeto Y, Kohara M, Miura T, Miura C, Yamaguchi S et al. (2008) Japanese eel follicle-stimulating hormone (fsh) and luteinizing hormone (lh): production of biologically active recombinant fsh and lh by Drosophila s2 cells and their differential actions on the reproductive biology. Biol Reprod 79: 938–946.
12. Garcia-Lopez A, de Jonge H, Nobrega RH, de Waal PP, van Dijk W et al. (2010) Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. Endocrinology 151: 2349–2360.
13. Ohta T, Miyake H, Miura C, Kamei H, Aida K et al. (2007) Follicle-Stimulating Hormone Induces Spermatogenesis Mediated by Androgen Production in Japanese Eel, *Anguilla japonica*. Biol Reprod 77: 970–977.
14. Garcia-Lopez A, Bogerd J, Granneman JC, van DW, Trant JM et al. (2009) Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150: 357–365.
15. Alam MA, Kobayashi Y, Hirai T, Nakamura M (2010) Isolation, characterization and expression analyses of FSH receptor in protogynous grouper. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 156: 364–371.
16. Chauvigne F, Verdura S, Mazon MJ, Duncan N, Zanuy S et al. (2012) Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. Biol Reprod 87: 35.
17. Sambroni E, Le Gac F, Breton B, Lareyre JJ (2007) Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. J Endocrinol 195: 213–228.
18. Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci U S A 88: 5774–5778.
19. Mazon MJ, Zanuy S, Carillo M, Gomez A (2011) Use of somatic gene transfer for studying gonadotropin actions on spermatogenesis in european sea bass (*Dicentrarchus labrax*). Indian J Sci Technol 4: 143–144.
20. Gomez JM, Weil C, Ollitrault M, Le Bail PY, Le Gac F (1999) Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 113: 413–428.
21. Loir M (1999) Spermatogonia of rainbow trout: II. in vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. Mol Reprod Dev 53: 434–442.
22. Loir M (1999) Spermatogonia of rainbow trout: I. Morphological characterization, mitotic activity, and survival in primary cultures of testicular cells. Mol Reprod Dev 53: 422–433.
23. Fostier A, Billard R, Breton B, Legendre M, Marlot S (1982) Plasma 11-Oxotestosterone and Gonadotropin During the Beginning of Spermiation in Rainbow-Trout (*Salmo-Gairdneri R*). General and Comparative Endocrinology 46: 428–434.
24. Rescan PY, Montfort J, Ralliere C, Le Cam A, Esquerre D et al. (2007) Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. BMC Genomics 8: 438.
25. Lopez F, Rougemont J, Loriod B, Bourgeois A, Loi L et al. (2004) Feature extraction and signal processing for nylon DNA microarrays. BMC Genomics 5: 38.
26. Rolland A, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ et al. (2009) Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. BMC Genomics 10: 546.

27. Chalmel F, Primig M (2008) The Annotation, Mapping, Expression and Network (AMEN) suite of tools for molecular systems biology. BMC Bioinformatics 9: 86.
28. Smyth GK, Michaud J, Scott HS (2005) Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21: 2067–2075.
29. Borg B (1994) Androgens in teleost fishes. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 109: 219–245.
30. Lubzens E, Young G, Bobe J, Cerdá J (2010) Oogenesis in teleosts: How fish eggs are formed. General and Comparative Endocrinology 165: 367–389.
31. van Bohemen CG, Lambert JG (1979) Steroidogenesis in the ovary of the rainbow trout, Salmo gairdneri [proceedings]. J Endocrinol 80: 37P–38P.
32. van den Hurk R, Lambert JG, Peute J (1982) Steroidogenesis in the gonads of rainbow trout fry (Salmo gairdneri) before and after the onset of gonadal sex differentiation. Reprod Nutr Dev 22: 413–425.
33. Fostier A, Le Gac F, Loir M (1987) Steroids in male reproduction. In: Idler DR, Crim LW, Walsh JM, editors. Reproductive physiology of fish. St-John's: University of Newfounland (CA). pp. 239–245.
34. Rolland AD, Lardenois A, Goupil AS, Lareyre JJ, Houlgatte R et al. (2013) Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. PLoS One 8: e53302.
35. Schulz RW, Lieburg M, Garcia-Lopez A, Dijk W, Bogerd J (2008) Androgens modulate testicular androgen production in African catfish (*Clarias gariepinus*) depending on the stage of maturity and type of androgen. Gen Comp Endocrinol 156: 154–163.
36. Planas JV, Swanson P, Rand-Weaver M, Dickhoff WW (1992) Somatolactin stimulates in vitro gonadal steroidogenesis in coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 87: 1–5.
37. Abel MH, Baker PJ, Charlton HM, Monteiro A, Verhoeven G et al. (2008) Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. Endocrinology 149: 3279–3285.
38. Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P et al. (2007) The conserved transcriptome in human and rodent male gametogenesis. Proc Natl Acad Sci USA 104: 8346–8351.
39. McLean DJ, Friel PJ, Pouchnik D, Griswold MD (2002) Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. Mol Endocrinol 16: 2780–2792.
40. Xia Y, Sidis Y, Schneyer A (2004) Overexpression of Follistatin-Like 3 in Gonads Causes Defects in Gonadal Development and Function in Transgenic Mice. Mol Endocrinol 18: 979–994.
41. Shen W, Park BW, Toms D, Li J (2012) Midkine promotes proliferation of primordial germ cells by inhibiting the expression of the deleted in azoospermia-like gene. Endocrinology 153: 3482–3492.
42. Zachow R, Uzumcu M (2007) The hepatocyte growth factor system as a regulator of female and male gonadal function. Journal of Endocrinology 195: 359–371.
43. Miura T, Miura C, Konda Y, Yamauchi K (2002) Spermatogenesis-preventing substance in Japanese eel. Development 129: 2689–2697.
44. Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW et al. (2011) Proteolytically Activated, Recombinant Anti-Mullerian Hormone Inhibits Androgen Secretion, Proliferation, and Differentiation of Spermatogonia in Adult Zebrafish Testis Organ Cultures. Endocrinology 152: 3527–3540.
45. Wang DS, Jiao B, Hu C, Huang X, Liu Z et al. (2008) Discovery of a gonad-specific IGF subtype in teleost. Biochemical and Biophysical Research Communications 367: 336–341.
46. Loir M, Le Gac F (1994) Insulin-like growth factor-I and -II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes. Biol Reprod 51: 1154–1163.
47. Berishvili G, Baroiller JF, Eppler E, Reinecke M (2010) Insulin-like growth factor-3 (IGF-3) in male and female gonads of the tilapia: development and regulation of gene expression by growth hormone (GH) and 17alpha-ethinylestradiol (EE2). Gen Comp Endocrinol 167: 128–134.
48. Li M, Wu F, Gu Y, Wang T, Wang H et al. (2012) Insulin-Like Growth Factor 3 Regulates Expression of Genes Encoding Steroidogenic Enzymes and Key Transcription Factors in the Nile Tilapia Gonad. Biol Reprod 86: 163.
49. Nelson SN, Van Der Kraak G (2010) The role of the insulin-like growth factor (IGF) system in zebrafish (*Danio rerio*) ovarian development. Gen Comp Endocrinol 168: 103–110.
50. Catizone A, Ricci G, Del Bravo J, Galdieri M (2006) Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. Journal of Endocrinology 189: 137–146.
51. Zhang N, Yeh HJ, Zhong R, Li YS, Deuel TF (1999) A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice. Proc Natl Acad Sci U S A 96: 6734–6738.

Table S1: Sequences of primers used for q-PCR experiments.

Gene symbol	Acc number	Forward primer	Reverse primer
rps15 (ref)	ACO08621	CCTGGGGGAGTTCTCTATCACCT	GGGATGAAACGGGAAGAATGTGT
fshr	AF439405	TCAGTCACCTGACGATCTGCAA	TCCTGCAGGTCCAGCAGAAACG
lhcgr	AF439404	CTTCTCAACCTCAATGAAATCTTC	GGATATACTCAGATAACGCAGCTT
igf1b	CX025953	GTGTGGAGACCGTGGATTTT	CACAATTCCCTTCCCTCTCA
igfbp6	DQ190459	GCTCAATAGTGTTCTGCGTGG	CTTGGAGGAACGACACTGCTT
star	AB047032	GAGTTGTTAGGGCAGAGAAC	CAACCCTTTAAATCTATGCTTA
hsd3b1	S72665	TACAGTGCCTGGAAGAGATCAGA	ACCCTGTGAAGCTCACTGTATAA
cyp11b2-2	AF217273	CTGGGACATGTGTCCAGGCA	CTGGATCCTGAAACACGTCA
fstl3	NM_001160487	ACCGCTGAAGTCCGAGTTGC	GCAGGTGGCTCTGTGGAGGT
amh	Q5XZF0	GGGAATAACCATGCTATCCTGCTTAA	CTCCACCACCTTGAGGTCCTCATAGT
inha	Q9DED3	CCAGCTCTGACTCTACCTGTGAT	CCTGGTTGTCGAGGGAGGATTG
inhba	D88463	AGGGCAAGGTGAACATACAG	CCTCGTGTCCACCATCTTCTC
inhbb	AB044566	GTTTCGCAGAGACAGATGAG	GTCACATACAGGTGCTGGTT
slc26a4	NM_001165915	CGGCACAAACATATACAGGAA	CCACCGTGACTCTCAATCGTTCT
vt1	CA375992	GAGGCTGGAGGAAGAGTGTG	TTCTGTTTGCTGGGTGACTG
mmp19	BX081049	AGTTCTGAGGGAGTGTGTGG	TGTTGTGAGGGATAGGAAGG
mdka	AF149802	CAGTGTTCTTGGTCTGCCTAA	CTTGGTCTTGACTCCAGTTGA
wisp1	BX868220	TTCAACTCAACAGGAAGAGC	AACTCAAGGAGGGTCAAGAT
ccnd1	CA374091	GTCCCTTTAACTGCAGAGAAGT	ATCGTGAGGTGTTACTGATGCT

PATHWAYS USED BY ANDROGENS OR FSH TO REGULATE TESTIS MATURATION

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Introduction:

In fish, gonadotropin hormones and sex steroids are known to trigger the initiation of testicular puberty and to promote final maturation of the gametes. In a series of studies, the molecular mechanisms involved were investigated through identifying androgen and FSH/LH modulated gene expression in the trout testis.

Methods:

A search for androgen dependent genes was initiated in pre-pubertal rainbow trout treated with physiological doses of androgens. Groups of seven to ten immature male trout were treated with testosterone (T) or 11-ketotestosterone (11KT) by implantation for 7-14 days. The treatment induced large increases in androgen plasma levels but these remained within the physiological range for adult male trout. FSH-regulated genes were also identified from trout testicular explants in early stages of development (I-II: spermatogonia only, III: initiation of meiosis) cultured in the presence of salmonid FSH at 500ng/ml. RNA was extracted from all testicular samples. In transcriptome studies using trout cDNA chips, the transcript accumulation for 9000 probes, corresponding to ~6000 non-redundant genes was measured. A limited number of candidate genes were also confirmed by *in situ* hybridisation and qPCR.

Results and discussion:

1) Up or down regulations of specific mRNA levels were observed after the androgen treatments or in response to LH /FSH. Early changes appeared to affect gene expression mainly in the somatic compartment, where receptors for these hormones have been described in fish. For many of these genes, significant developmental expression patterns during the annual maturation of the trout testis were observed. Some changes also corresponded well with the natural increases in circulating hormone levels during the reproductive cycle, reinforcing the idea that the hormone induced changes that we describe are *physiologically* relevant.

2) Conversely to mammals, FSH and LH had similar effects on one third of gonadotropin regulated transcripts. These genes are candidate targets for FSH physiological action during the pubertal transition since only FSH is detected at these stages of testis development. Interestingly, we also found that about 80 candidates appeared regulated exclusively or much more efficiently by FSH (rather than by LH) which should help to clarify the respective roles of the 2 gonadotropins in fish.

3) Data mining of the candidates revealed that hormones affected somatic genes involved in germ cell sustaining proliferative activity or evading growth suppressors, or differentiation.

- In prepubertal testis FSH and/or androgens affected the expression of several transcripts coding for paracrine factors of somatic origin, like GSDF, AMH, inhibin, ,Follistatin, BMP7, which are all potentially involved in germ cell proliferation or differentiation
- In addition to modifying gene expression in supporting somatic cells, androgen treatment resulted in a shift in germ cell gene expression profiles, with a decrease in several genes preferentially expressed in spermatogonia (like *noc2l*) and an increase in transcripts with a meiotic/post meiotic profile (like *morn3, rsph3, bty*). This reinforces the involvement of androgens in the transition from spermatogonia towards more differentiated germ cells expression profiles.
- Two transcripts encoding **G2/mitotic-specific cyclins** were up regulated by FSH/LH; one, annotated ccnb3 is known as involved in early meiosis in mouse; the second one, annotated *ccnb1* and expressed in late germ cells responded better to FSH, with a stronger effect in stage III (in contrast the G1/S-specific cyclin-E1 was found down regulated by the gonadotropins).
- Gonadotropins up regulated some germinal or somatic genes encoding intracellular factors known to take part, directly or indirectly, in germ cell fate, for example *nanos3* (FSH and LH), *dmrt1* (FSH and LH) or *sox8* (LH) and also affected genes encoding factors involved in other developmental processes like apoptosis (*casp8*, *faf1* and *clu1*) or testicular fluid homeostasis (iron/ion transporters *slc30a1*, *fth1*).
- FSH regulated several transcripts encoding proteins involved in microtubule and cytoskeleton rearrangement (calponin2 *cnn2*, desmin *des*, cytokeratins *krt18*, *krt8*, microtubule-associated protein *mapre1*) and proteins of the extracellular matrix (collagen *col1a1*, *col1a2*, proteases *mmp19*, *mmp9* and protease inhibitors, *timp2*) important for cell adhesion and migration and cell junctions.

4) The steroidogenic pathway was of course affected by the hormone treatments: the gonadotropins were generally stimulatory (*star, hsd3b1*), while *in vivo* treatment with high levels of androgens were inhibitory

(indirectly, through an FSH inhibition, or directly, via a short loop feed-back on steroidogenic enzymes).

5) Furthermore, a specific group of the regulated transcripts probably take part in the morpho-functional changes in the testis and sperm duct during spermiation in trout. We found that sex steroids possibly control these genes involved in the regulation of water exchanges (*aqp1, aqp4, vt1*) and in proton and potassium regulation in seminiferous tubules (*cahz, vt1, slc26a4, atp1b1, lgi1)*, and are therefore relevant concerning sperm maturation and excretion.

Conclusions:

We provide meaningful information on a large set of transcripts implicated in the testicular somatic cell response to gonadotropin and/or androgens. These include growth factors, extra-cellular matrix components and intracellular pathways which are potentially involved in the mechanisms by which reproductive hormones direct germ cell renewal or differentiation at puberty, then sperm maturation and excretion at spawning time in adult fish.

Fsh and Lh have both common and distinct effects on testicular gene expression in rainbow trout

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LIFECYCLE

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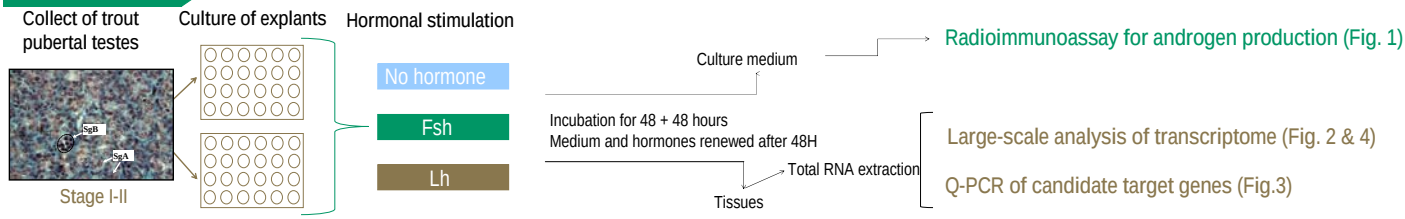
Context: Gonadotropins are essential for the control of spermatogenesis. In fish, the respective roles of Fsh and Lh remain to be clarified. Indeed the presence of Fshr on Leydig cells has been demonstrated in several species and Fsh is a potent stimulator of steroidogenesis. Fsh is thought to be the major gonadotropin in the initial stage of testis maturation. However the molecular mechanisms underlying the respective action of Fsh and Lh remain unknown and the pathways that relay the action of Fsh in fish at puberty are poorly understood.

Objectives: -To identify genes regulated by Fsh and disclose its role in early spermatogenesis

- To discriminate between Fsh and Lh effects

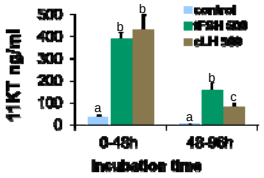
Method

In vitro stimulation of testicular tissue explants by purified salmonid gonadotropins (100 to 500 ng/mL)



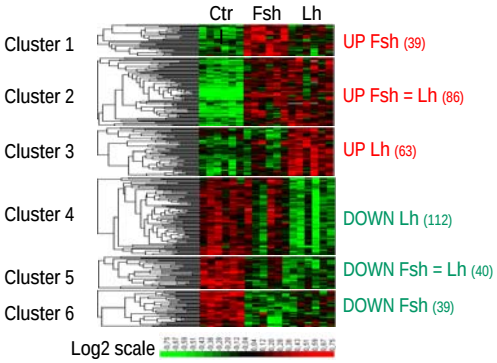
Results

Fig.1 11KT production in culture media



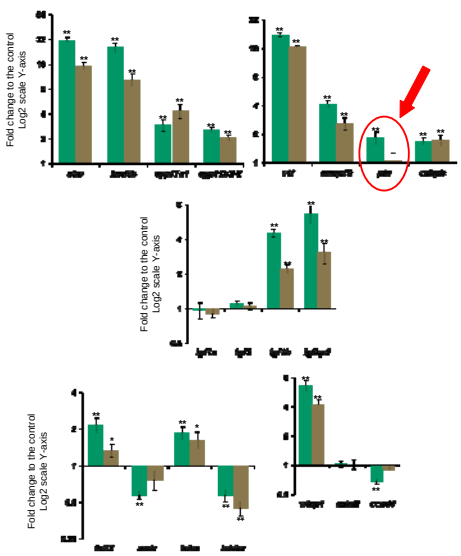
- ✓ Both Fsh and Lh stimulate 11KT production
- ✓ Gonadotropin responsiveness of the cultured testes is maintained over the culture duration

Fig.2 Transcriptome changes after gonadotropin treatments (number of genes).



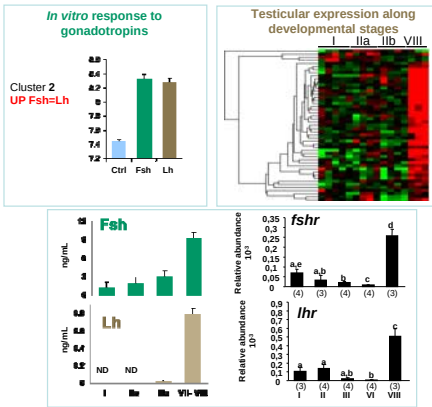
Fsh and Lh have
- ✓ common effects : clusters 2 and 5
- ✓ and distinct effects : clusters 1, 3, 4 and 6

Fig.3 q-PCR for candidate target genes.



- ✓ Q-PCR validated the microarray data for both common and preferential effect of Fsh and Lh on gene expression
- ✓ *Ptn* is exclusively up regulated by Fsh

Fig.4 LH= FSH UP regulated genes : expression during the reproductive cycle.

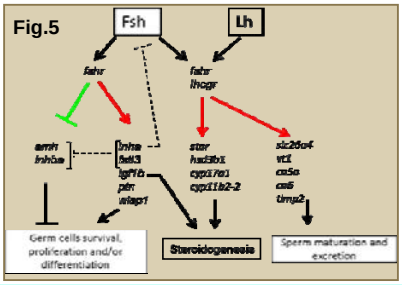


- ✓Somatic expression for a majority of genes
- ✓Most UP-regulated genes are highly expressed at stage VIII of the reproductive cycle,
- ✓When Fsh and Lh plasma levels and expression of their receptors in testis are highest

Conclusions

Fsh and Lh had both common and distinct effects on testicular gene expression. Beside Fsh action on the steroidogenic pathway, we identified original Fsh regulated genes that could be relevant physiological mediators of Fsh action on early spermatogenesis. Some of these genes belong to the Igf pathway (*igf1b*, *igfbp6*), the Tgfb pathway (*amh*, *inha*, *inhba*, *fstl3*), the Wnt pathway (*wisp1*) and pleiotrophin (Fig. 5)

Fig.5



A number of genes up-regulated *in vitro* by both Fsh and Lh (*slc26a4*, *ca5a*, *ca6*, *timp2* and *vt1*) are also found naturally and strongly increased at the end of the reproductive cycle, when both gonadotropins and their receptors reach their maximum secretion and expression levels, respectively. It shows that, in addition to Lh, Fsh may have important functions during final stages of sperm maturation and transportation.

Physiologie Génomique Poissons

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ALIMENTATION AGRICULTURE ENVIRONNEMENT

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DISCUSSION GENERALE

I. L'action de Fsh et de Lh passe préférentiellement par leurs récepteurs respectifs

Les connaissances acquises sur l'évolution de l'expression de Lhr et de Fshr au cours du cycle et sur la spécificité des liaisons hormone-récepteur, mises en relation avec les variations des niveaux de Fsh et de Lh circulantes apportent un éclairage important dans la compréhension des rôles biologiques de Fsh et de Lh.

Nos travaux chez la truite visant à caractériser les récepteurs des gonadotropines sur les plans moléculaire et fonctionnel ont décrit la présence de deux récepteurs distincts montrant des similitudes avec ceux des vertébrés supérieurs, mais aussi des différences en termes de déterminants structurels. Les études fonctionnelles *in vitro* montrent une spécificité relative forte des 2 récepteurs vis-à-vis de leur ligand, y compris pour Lhcgr qui n'est activé qu'à des doses très élevées de Fsh (> 800 ng/ mL, doses jamais rencontrées à aucun moment du cycle). Depuis notre étude, d'autres travaux ont été entrepris chez d'autres espèces de poissons: bar : (Rocha *et al.* 2007) ; flétan : (Kobayashi *et al.* 2008) ; saumon atlantique : (Andersson *et al.* 2009); sole sénégalaise : (Chauvigne *et al.* 2012) et arrivent à des conclusions contradictoires. Quand des hormones hétérologues sont utilisées, un des 2 récepteurs présente des interactions croisées avec l'hormone non apparentée : Lhcgr vis-à-vis de la FSH bovine chez le bar, Fshr vis-à-vis de la Lh de bar chez le flétan et la sole sénégalaise. Chez le saumon atlantique, la Lh active aussi le récepteur Fshr mais avec une potentialité 6 fois inférieure à celle de la Fsh. Pour comprendre les causes possibles des interactions croisées entre ligands et récepteurs, un groupe a testé les capacités d'activation des hormones recombinantes de 3 espèces de poissons, appartenant à 3 ordres différents, sur leurs récepteurs apparentés et non apparentés (Aizen *et al.* 2012). Ils ont montré qu'il existe des interactions croisées entre un récepteur d'une espèce et des gonadotropines apparentées mais d'espèces différentes. Ainsi, le récepteur Lhcgr de tilapia (taLhcgr) peut être activé par la Lh recombinante de tilapia (taLh), mais aussi par les Lh recombinantes d'autres espèces (hCG, eLh et trLh). A l'inverse, pour un récepteur donné, il peut exister des interactions avec les gonadotropines réciproques hétérologues.

C'est le cas, par exemple, pour le récepteur Lhcgr d'anguille qui est activé par l'eLh mais aussi par la FSH humaine. L'analyse des structures primaire et tridimensionnelle des domaines fonctionnels des hormones et des récepteurs impliqués dans leur liaison peut en partie expliquer ces interactions croisées. Par exemple, l'activation du récepteur eLhcgr par la FSH humaine serait possible du fait de la présence, dans le motif LRR1, d'un résidu histidine en position 64 dont la taille autorise la liaison avec la FSH. Chez le tilapia, le récepteur de Fsh possède dans sa séquence une insertion dans le domaine riche en leucine (LRR), due à la duplication d'un exon, qui pourrait affecter la liaison et l'interaction de Fsh au récepteur (Aizen *et al.* 2012). De même, chez les poissons, Fshr ne possède pas, dans sa partie N-terminale, un domaine NCR typique constitué de 4 résidus cystéine conservés chez les homologues mammaliens (Sambroni *et al.* 2007; Levavi-Sivan *et al.* 2010). Selon les espèces, 1 ou 2 résidus sont seulement retrouvés, ce qui pourrait entraîner une conformation tridimensionnelle différente plus favorable à des interactions non spécifiques.

Finalement, l'idée d'une spécificité limitée du Fshr chez les poissons reste défendue par quelques auteurs (Kobayashi *et al.* 2008; Andersson *et al.* 2009). Cependant, cette idée repose principalement sur des données acquises *in vitro* en système d'expression transitoire. Or, nous avons montré que le contexte cellulaire dans lequel les récepteurs sont exprimés influence fortement les interactions hormone-récepteur (Voir partie 1, données complémentaires). Des tests utilisant des préparations membranaires et des hormones hautement purifiées ou recombinantes avec une activité biologique élevée et comparable entre les 2 gonadotropines permettraient de trancher cette question.

En conclusion, même si une interaction croisée entre récepteurs et hormones ne peut pas être totalement écartée, elle semble très improbable dans les conditions physiologiques *in vivo*, vu les doses ou la nature des hormones nécessaires pour activer le récepteur réciproque.

La probabilité d'une réaction croisée de Fsh sur les récepteurs de Lh ne peut donc pas expliquer le fort potentiel de Fsh à stimuler la stéroïdogenèse. Des travaux anciens proposaient une activité stéroïdogène des cellules somatiques intratubulaires. Une autre hypothèse envisagée est que les récepteurs de Fsh sont aussi exprimés dans les cellules stéroïdogènes de l'interstitiel. Cette hypothèse a été vérifiée chez plusieurs espèces de téléostéens (Ohta *et al.* 2007; Garcia-Lopez *et al.* 2009; Garcia-Lopez *et al.* 2010; Chauvigne *et al.* 2012). Des travaux au sein de notre équipe n'ont pas permis de conclure quant à la

localisation cellulaire des récepteurs chez la truite. Si la présence des récepteurs de Fsh sur les cellules de Leydig est confirmée chez la truite, on pourra conclure à une action directe de Fsh sur les cellules stéroïdogènes de façon similaire à Lh et cela expliquerait que les 2 hormones ont la même aptitude, à certaines phases du cycle, à stimuler la production de stéroïdes. Cette caractéristique constituerait chez certaines espèces saisonnières, une voie importante de l'action de Fsh, en l'absence de Lh circulante en début de cycle, pour assurer la production d'androgènes nécessaire à la progression de la spermatogenèse.

Le récepteur de Lh est généralement décrit dans l'interstitiel. Chez la sole sénégalaise où transcrits et protéines ont été localisés, Lhcgr n'est présent que sur les cellules de Leydig (Chauvigne *et al.* 2012). Une seule étude réalisée chez le poisson zèbre décrit son expression dans les cellules de Leydig mais aussi dans les cellules de Sertoli (Garcia-Lopez *et al.* 2010). Les auteurs font l'hypothèse que cette expression inattendue pourrait être due au mode de différenciation gonadique du poisson zèbre qui est une espèce à gonochorisme indifférencié : les individus passent par un stade ovarien précoce et, environ 30 jours après fécondation, chez certains individus, les ovaires évolueront en testicules tandis que chez les autres, ils continueront leur développement en ovaires matures. Comme les cellules de la granulosa expriment à la fois Fshr et Lhcgr, il est possible que la coexpression de *fshr* et de *lhcgr* soit liée au stade de développement femelle transitoire et perdure dans les cellules de Sertoli de poisson zèbre mâle, représentant ainsi une particularité de cette espèce. Par ailleurs, l'étude n'a porté que sur le transcrit et pas sur la protéine qui pourrait ne pas être produite. Les données actuelles indiquent donc que le Lhcgr n'est généralement pas présent sur les cellules de Sertoli chez les poissons. La localisation du récepteur de Lh serait ainsi très similaire entre les espèces pisciaires et mammaliennes, contrairement au récepteur de Fsh.

II. Expression et régulation des récepteurs

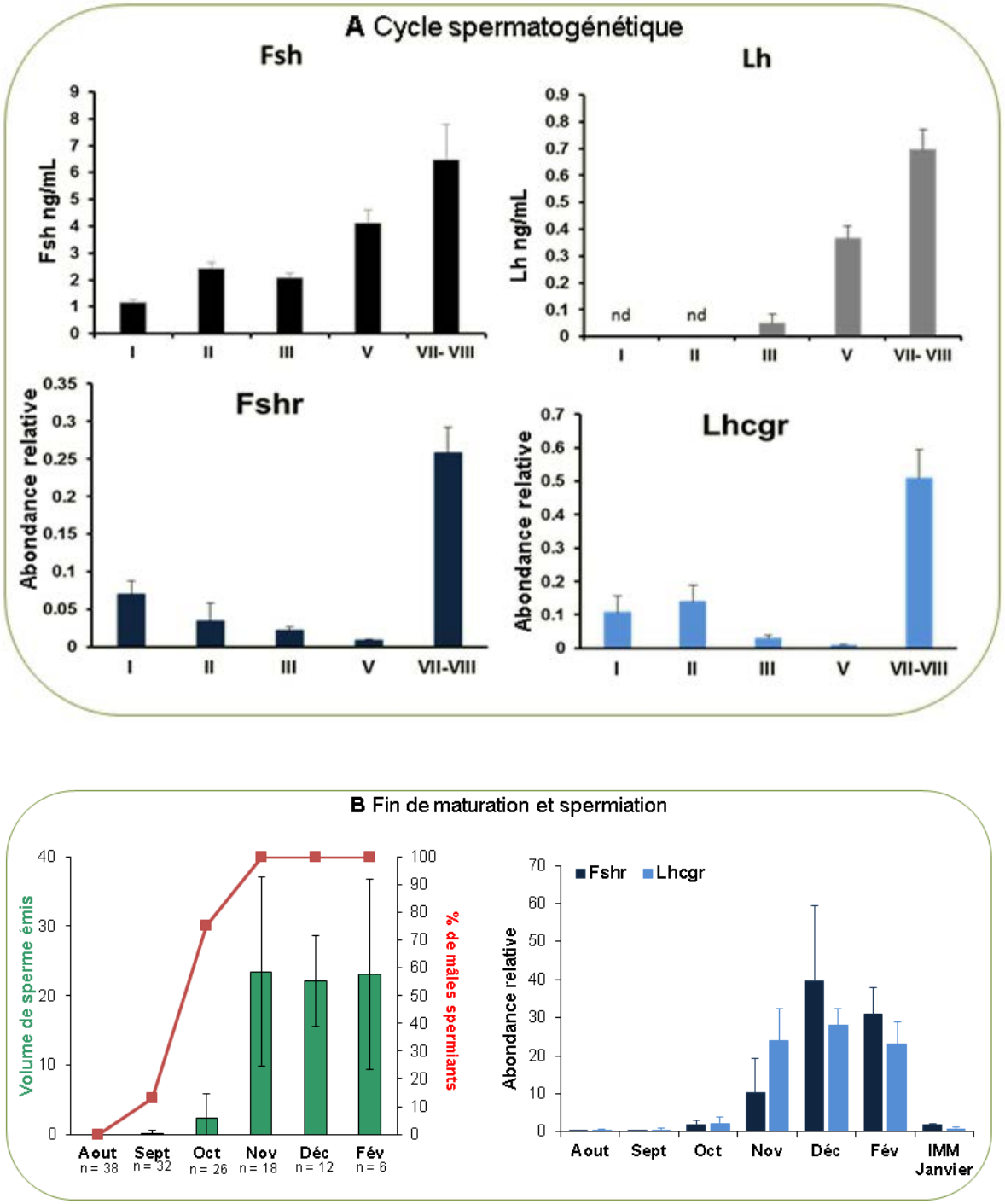

Figure 10 : **A** : Profils de sécrétion de Fsh et Lh et expression des récepteurs Fshr et Lhcgr au cours du cycle de reproduction de la truite (Gomez et al., 1999 et Sambroni et al., 2007). **B :** Expression de Fshr et de Lhcgr avant et pendant la période de spermiation chez la truite (Bellaiche et al., non publié).

1. Profil d'expression au cours du cycle

L'étude de l'expression des récepteurs au cours du cycle de reproduction chez la truite ne permet pas d'élaborer des hypothèses fortes quant à leur rôle respectif au cours de la spermatogenèse. En effet, contrairement aux profils de sécrétion de Fsh et de Lh qui présentent des différences importantes chez la truite (figure 10A), les profils d'expression des 2 récepteurs se révèlent très similaires entre eux. Les transcrits *fshr* et *lhcgr* sont détectés à tous les stades étudiés, varient peu au cours de la spermatogénèse et présentent tous les 2 une forte élévation en fin de cycle. Cela amène une réflexion : un facteur déterminant dans le contrôle gonadotrope de la spermatogenèse, au moins en début de cycle, serait la sécrétion et les concentrations circulantes des gonadotropines plutôt que les récepteurs eux-mêmes.

Des résultats complémentaires (figure 10B) précisent que l'expression des 2 récepteurs s'élève fortement en novembre chez une souche de truite à reproduction automnale, simultanément avec les entrées en spermiation et l'élévation du volume de sperme émis suggérant un rôle particulier à ce stade de développement. Nos résultats diffèrent de ceux obtenus par une autre équipe sur la même espèce, *Oncorhynchus mykiss*, qui décrivent pour *fshr*, une augmentation de son expression coïncidant avec une forte activité méiotique, puis des fluctuations de faible amplitude, y compris pendant la période de spermiation (Kusakabe *et al.* 2006). Le profil d'expression de *lhcgr* est lui aussi différent en ce qu'il présente une augmentation continue depuis l'entrée en méiose jusqu'à la spermiation. Cette divergence semble tenir à la souche printanière utilisée dans l'hémisphère sud. Chez un autre salmonidé, le saumon atlantique, les transcrits des récepteurs Fshr sont plus abondants que ceux de Lhcgr dans les gonades immatures (Maugars *et al.* 2008). Cependant les niveaux d'expression des 2 transcrits sont tous les 2 maxima à la spermiation. Cette élévation peut résulter de 2 phénomènes : d'une part, la régulation hormonale de l'expression des transcrits et d'autre part, l'augmentation du nombre de cellules qui expriment ces transcrits.

2. Régulation de l'expression

L'incubation d'explants testiculaires en présence de Fsh et de Lh, à moyen terme, résulte en une augmentation des transcrits des récepteurs, *fshr* et *lhcgr*. Chez l'anguille, un extrait hypophysaire de saumon induit une augmentation significative des transcrits *fshr* et *lhcgr*, suggérant l'implication des gonadotropines dans la régulation de leur expression (Jeng *et al.* 2007). La régulation par les gonadotropines de leurs propres récepteurs témoigne d'une boucle de régulation positive qui participerait à un système d'amplification efficace des effets

trophiques des gonadotropines. De plus, nous montrons que cette régulation est dépendante de la production de stéroïdes, puisqu'elle est supprimée en présence de trilostane. Par ailleurs, chacune des gonadotropines régule positivement les 2 récepteurs, ce qui est tout à fait compatible avec le mode d'action des hormones via les stéroïdes. Bien que notre étude ne nous permette pas d'incriminer un stéroïde en particulier, nos données sont cohérentes avec l'augmentation, induite *in vitro* par les androgènes, des niveaux de *fshr* et *lhcgr* dans le testicule de poisson-chat africain (Schulz *et al.* 2008). La forte augmentation de l'expression des transcrits *fshr* et *lhcgr* observée chez la truite à la fin du cycle reproducteur (Sambroni *et al.* 2007)(et Figure 10B) peut s'expliquer par les régulations mises en évidence *in vitro*, puisque la fin du cycle est caractérisée par des taux plasmatiques élevés de gonadotropines et de stéroïdes sexuels (androgènes et progestagènes) (Gomez *et al.* 1999).

III. Rôle physiologique des récepteurs

Chez la truite, puisque les gonadotropines interagissent préférentiellement avec leurs récepteurs respectifs, l'auto-immunisation dirigée contre le récepteur de Fsh ou de Lh est un moyen intéressant pour étudier *in vivo* le rôle physiologique respectif des gonadotropines et de leurs récepteurs dans les fonctions testiculaires. Cette méthode, moins coûteuse et plus rapide à mettre en place, a été préférée aux méthodes de KO de gènes qui n'étaient pas développées chez les poissons quand le travail a été initié.

Nous avons montré que la vaccination contre le récepteur de Fsh (anti-FSHR) chez la truite mâle pré pubère, inhibe fortement l'élévation des taux plasmatiques de testostérone et de 11KT, effet particulièrement visible en fin de cycle. Ces résultats confirment que le signal Fsh/Fshr est bien impliqué dans la régulation de la stéroïdogenèse. La vaccination contre Fshr empêche l'entrée en maturation d'une proportion faible mais significative des mâles, alors que l'inactivation du signal Lh/Lhcgr n'a pas eu cet effet. Cela montre que seul le signal Fsh/Fshr est nécessaire pour le développement initial des cystes en spermatogenèse active. Cependant, les 2 vaccinations engendrent une diminution tardive des niveaux plasmatiques d'androgènes. Cela suggère que l'action spécifique du signal Fsh/Fshr ne passe pas seulement par la production de stéroïdes, mais implique des facteurs régulés plus directement par Fsh, importants pour le contrôle des phases précoces du cycle. Du reste, les données sur les effets comparés de Fsh et de Lh sur l'expression testiculaire des gènes en début de cycle appuient très fortement cette allégation. En effet, de nombreux gènes régulés par Fsh, mais pas par Lh, le sont indépendamment de la production de stéroïdes (**III** et **IV**).

Parmi ces gènes, on trouve *amh* et *igf1b* qui sont impliqués dans la prolifération et la différenciation des spermatogonies A.

Quand l'immunisation est initiée à un stade plus tardif du cycle sexuel, seule la double vaccination contre les deux récepteurs à la fois (anti-FSHR+LHR) entraîne un retard de développement de la spermatogenèse, essentiellement attribuable à un arrêt des cellules germinales au stade pré méiotique. Cela suggère une coopération entre les 2 voies de signalisation dans le contrôle de la progression de la méiose. Comme chez le pré pubère, la vaccination entraîne une diminution des taux plasmatiques de testostérone. Chez la truite, il a été montré que l'administration *in vivo* d'androgènes à des truites mâles immatures a tendance à stimuler l'expression de gènes spécifiques des cellules germinales impliqués dans la différenciation méiotique (Rolland *et al.* 2013). On peut donc proposer que les androgènes sont les médiateurs majeurs de l'action des gonadotropines sur la progression de la méiose. Ce mécanisme de régulation est à rapprocher de la situation chez la souris où les mâles KO pour LH/CGR ou pour les récepteurs aux androgènes (ARKO) sont totalement stériles à cause de l'arrêt de la spermatogenèse au cours de la méiose, démontrant clairement le rôle majeur des androgènes (pour revue, (Zhou 2010).

Notre dispositif expérimental nous a permis de mesurer certains indicateurs du bon fonctionnement testiculaire : taille des testicules, présence de chaque type de cellules germinales, avancement de la maturation spermatogénétique et stéroïdogenèse, mais ne nous informe pas sur les cellules de Sertoli. Or, on peut spéculer qu'une part importante de l'action de Fsh s'effectue sur les cellules de Sertoli. On sait chez les mammifères que la FSH est le facteur majeur qui contrôle l'expansion de la population de cellules de Sertoli qui a lieu au cours du développement fœtal et cesse au cours de la première vague de la spermatogenèse, lorsque les spermatocytes primaires se multiplient activement. Chez les poissons (poisson-chat africain et tilapia), la prolifération des cellules de Sertoli accompagne la prolifération rapide des spermatogonies permettant l'accroissement des cystes lié à la multiplication des cellules germinales (Schulz *et al.* 2005). L'étude de la prolifération des cellules de Sertoli chez les animaux dont on a bloqué spécifiquement le signal Fsh/Fshr pourrait fournir des arguments pour établir un lien fonctionnel entre l'élévation des taux plasmatiques de Fsh au début du cycle et la prolifération des cellules de Sertoli.

Outre l'intérêt d'acquérir des connaissances fondamentales sur le rôle spécifique de chacun des récepteurs, notre objectif majeur était le développement pour l'aquaculture, d'une méthode de vaccination efficace pour rendre les animaux d'élevage stériles ou au minimum

retarder la puberté chez ceux qui atteignent la maturité sexuelle trop précocement, au détriment de la croissance et de la qualité de la chair. En dépit de l'utilisation d'un adjuvant et de la fréquence des injections, les vaccinations n'ont induit qu'une faible réponse immunitaire chez la truite, phénomène qui pourrait être lié à la température d'élevage qui avoisine les 10°C. Une alternative intéressante à la vaccination classique pourrait être les vaccins ADN qui présentent un double avantage : ils sont peu coûteux à produire et sont très efficaces. Autre avantage non négligeable du vaccin ADN : une seule inoculation suffit. Depuis quelques années, cette méthode de vaccination s'est révélée efficace en aquaculture pour protéger les poissons contre différents rhabdovirus (Heppell *et al.* 2000; Lepa *et al.* 2010).

IV. Mécanisme d'action des gonadotropines

Dans le but de différencier les rôles respectifs de Fsh et de Lh dans le contrôle des fonctions testiculaires, nous nous sommes intéressés à leurs actions moléculaires en étudiant les modifications du transcriptome testiculaire induites *in vitro* par un traitement avec de la Fsh et de la Lh. Chez de nombreuses espèces saisonnières, Fsh est la seule gonadotropine sécrétée dans le plasma au cours des phases précoces du cycle. De plus Fsh stimule fortement la production de stéroïdes. Or, chez l'anguille japonaise la 11KT est capable à elle seule d'induire toutes les étapes de la spermatogenèse dans des testicules immatures, depuis la prolifération des spermatogonies jusqu'à la spermiogénèse (Miura *et al.* 1991; Ohta *et al.* 2007). L'action majeure de Fsh dans la régulation de la spermatogenèse est-elle limitée à la stimulation de la production de stéroïdes par les cellules de Leydig, comme pouvaient le laisser suggérer les travaux chez l'anguille ? Ou existe-t-il une action de Fsh sur les cellules de Sertoli qui ne passerait pas par la production de stéroïdes ? C'est à cette question que nous avons cherché à répondre en réalisant des incubations d'explants testiculaires en présence de Fsh seule ou en combinaison avec le trilostane, un inhibiteur connu de la 3 β-hydroxy stéroïde déshydrogénase.

A. Forces et limites de notre démarche

Pour l'analyse du transcriptome nous avons utilisé des membranes nylon à ADNc de truite qui comportent environ 6000 gènes non redondants et qui, au sein de notre groupe, ont été utilisées dans 4 expériences indépendantes conçues pour identifier des gènes ou des réseaux de gènes régulant des processus clés de la spermatogenèse :

- *in vivo*, au cours du cycle de reproduction et dans des populations enrichies de cellules germinales (Rolland *et al.* 2009)
- *in vivo*, après un apport exogène d'androgènes chez le pré pubère (Rolland *et al.* 2013)
- *in vitro* après traitement d'explants testiculaires en culture par Fsh et Lh (Sambroni *et al.* 2013a)
- *in vitro* après traitement d'explants testiculaires en culture par Fsh seule ou en combinaison avec le trilostane (Sambroni *et al.* 2013b)

L'exploration de cet ensemble cohérent de données nous a permis d'en extraire des informations sur la pertinence biologique des régulations que nous avons identifiées. Ainsi, l'intégration des données obtenues dans les études I et III, a permis de vérifier la cohérence entre le profil de sécrétion des gonadotropines (Gomez *et al.* 1999), les régulations des gènes par Fsh et Lh (mon étude) et le profil d'expression de ces mêmes gènes au cours du cycle (Rolland *et al.* 2009). Nous avons aussi pu associer la localisation de l'expression d'une majorité de gènes régulés par Fsh aux cellules somatiques. De même, par l'analyse intégrative des données obtenues dans les études II, III et IV, nous avons montré que les gènes préférentiellement régulés par Fsh et non par Lh étaient peu sensibles au trilostane, alors qu'une majorité des gènes régulés à la fois par Fsh et Lh étaient sensibles au trilostane, donc dépendants de la production de stéroïdes. Ce mode d'action des gonadotropines impliquant la production de stéroïdes a été conforté par le fait que, *in vivo*, un certain nombre de gènes ont été trouvés régulés par les androgènes.

Grâce au soin porté à l'annotation des clones, notamment en recherchant les gènes orthologues dans les génomes de poissons modèles disponibles (*Danio rerio*, *Oryzias latipes*, *Gasterosteus aculeatus*, et *Takifugu rubripes*), 90 % des clones truite ont pu être associés à des gènes orthologues ou au moins à des gènes codant des protéines appartenant à la même famille. L'annotation a pu être enrichie avec les termes de « GeneOntology » à partir de la base Ensembl, version 52 (Rolland *et al.* 2009).

Des incubations de 96 heures ont été clairement favorables pour identifier un grand nombre de gènes régulés par les gonadotropines ; cependant cette durée d'incubation ne permet peut-être pas de mettre en évidence les réponses les plus précoces induites par les gonadotropines et donc d'identifier les réponses les plus primaires aux hormones. Chez le rat, la FSH induit fortement l'expression de plus de 200 gènes dans les cellules de Sertoli au bout de 2 heures seulement de traitement (McLean *et al.* 2002). Le délai de réponse nécessaire pour observer les effets de la Fsh sur l'expression des gènes chez la truite pourrait être la

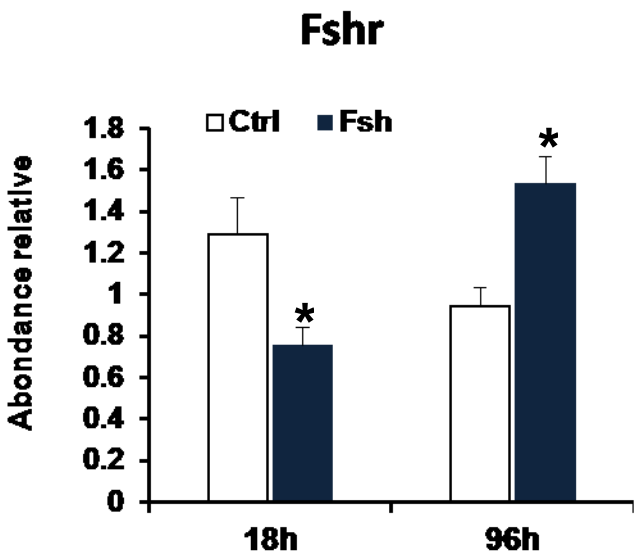

Figure 11 : Abondance relative des transcrits *fshr*, mesurée par qPCR, dans du tissu testiculaire après des incubations de 18h et de 96h en présence de Fsh. Les étoiles indiquent une différence statistique avec le contrôle.

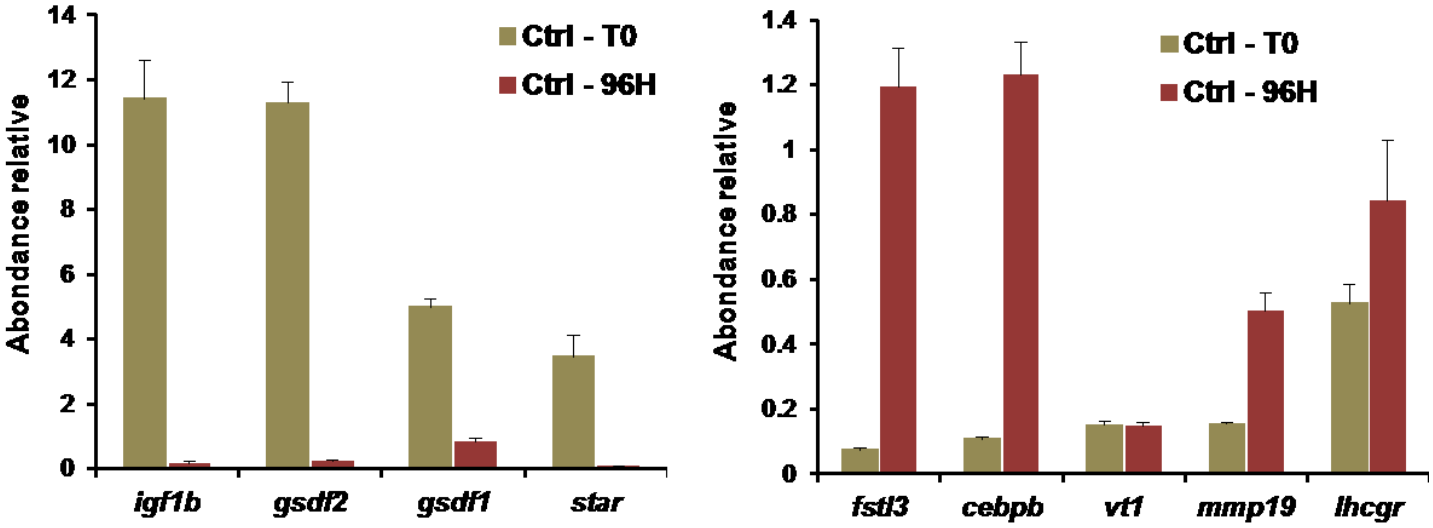

Figure 12 : Abondance relative de différents transcrits, mesurée par qPCR, dans du tissu testiculaire avant la mise en culture (T0) et après une incubation de 96h à 12°C, sans traitement hormonal (Ctrl).

conséquence d'un métabolisme ralenti du fait d'une température d'incubation plus basse (12°C). Dans notre étude, les réponses géniques observées sont vraisemblablement pour une part, dues à l'action « directe » des gonadotropines, via la voie de transduction du signal des récepteurs, sur le promoteur des gènes régulés, et pour une large part, le résultat de cascades d'activation de gènes qui impliquent notamment des facteurs de régulation de la transcription. On ne peut pas non plus exclure, dans de rares cas, des régulations qui s'inversent en fonction du temps. C'est le cas du gène *fshr* qui est réprimé par Fsh à 18h mais stimulé à 4 jours (Figure 11). Cela peut refléter une action primaire négative de Fsh sur ses récepteurs puis une action indirecte positive et dominante par les stéroïdes produits.

Il faut aussi noter qu'un grand nombre de gènes voient leur expression fortement modifiée, à la hausse ou à la baisse, au cours de la culture, ce qui peut être lié à une souffrance du tissu mais le plus souvent, témoigne, comme le montrent nos résultats, de l'existence de régulations endocrines *in vivo* qui sont absentes *ex vivo* dans nos conditions contrôles de culture (Figure 12).

Nous n'avons pas choisi de nous intéresser aux voies de signalisation, ni aux actions post-transcriptionnelles de Fsh, bien que ces mécanismes participent fortement au mode d'action de Fsh – au moins chez les mammifères – et peuvent expliquer les actions pléiotropiques de Fsh au sein d'une même cellule. Par exemple, chez le rat, il a été montré que les activités biologiques de FSH peuvent mobiliser des voies de transduction du signal différentes en fonction de l'état de développement des cellules de Sertoli, cibles naturelles de FSH (Crepieux *et al.* 2001). Dans notre étude nous avons mesuré l'effet des gonadotropines sur le taux d'accumulation des transcrits qui résulte à la fois de l'activité de transcription et de la stabilité des messagers. Cependant, la Fsh pourrait aussi avoir un rôle dans le contrôle de la traductibilité des transcrits. Des travaux récents ont montré que FSH pouvait contrôler l'efficacité de traduction d'ARNm pré existants. Les mécanismes qui contrôlent la machinerie traductionnelle impliquent des changements dans la phosphorylation des facteurs d'initiation et d'élongation de la traduction, mais aussi des interactions et/ou des dissociations protéine – protéine (Musnier *et al.* 2012). Aborder ces aspects permettrait de savoir si Fsh et Lh activent les mêmes voies de transduction du signal, en particulier dans les cellules de Leydig.

Malgré le nombre limité de gènes (environ 6000 gènes non redondants) présents sur nos microarrays, notre analyse du transcriptome testiculaire a permis de retrouver certains facteurs déjà connus chez les rongeurs pour être régulés par Fsh (par exemple, Star, Dmrt1, Inha),

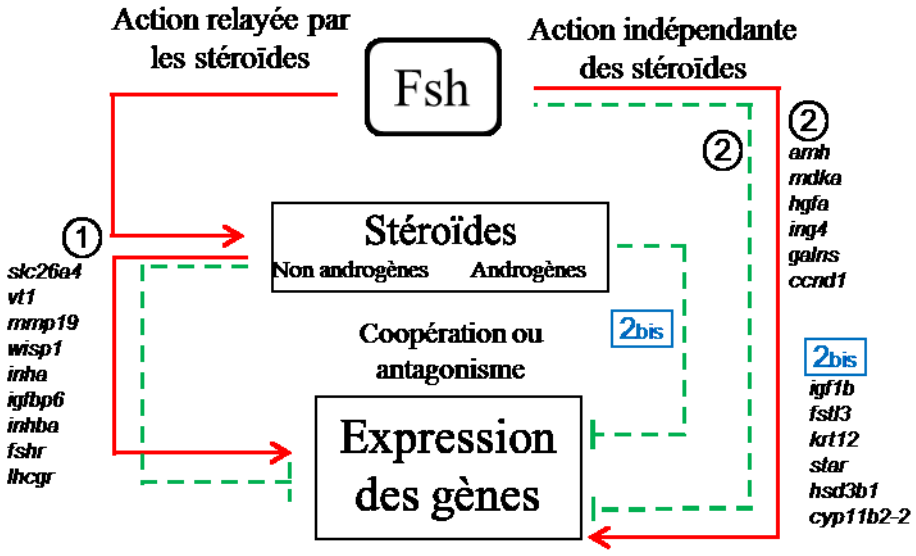

Figure 13: Résumé des mécanismes sous-tendant l'action de Fsh sur l'expression des gènes dans le testicule de truite. 1- l'action primaire de Fsh est de stimuler les cellules stéroïdogènes pour produire les stéroïdes qui en retour régulent l'expression des gènes. 2- Fsh exerce des effets régulateurs spécifiques, indépendamment de la production de stéroïdes. 2 bis- dans certains cas de régulation positive par Fsh, les stéroïdes pourraient avoir, indépendamment, des effets antagonistes. Les flèches rouges indiquent des effets stimulateurs tandis que les lignes vertes indiquent des effets inhibiteurs.

validant ainsi nos résultats. Mais notre étude a surtout permis d'identifier de nouveaux acteurs dont le rôle potentiel dans la spermatogenèse est discuté mais reste à démontrer chez la truite.

B. Comment les 2 gonadotropines régulent-elles l'expression génique dans le testicule (III et IV)

Pour la première fois à grande échelle chez un poisson, nous avons identifié des gènes cibles de l'action des gonadotropines et nous avons pu mettre en évidence une régulation commune aux 2 gonadotropines pour un ensemble de gènes et des régulations distinctes, voire opposées, pour d'autres groupes de gènes.

Nous avons pu mettre en évidence 2 mécanismes d'action de la Fsh : un mécanisme d'action indirect relayé par la production de stéroïdes et un mécanisme d'action indépendant de la production de stéroïdes (Voir figure 13). Notre étude a aussi révélé des gènes très probablement impliqués dans l'action des gonadotropines sur la maturation testiculaire.

a. Gènes régulés de façon similaire par Fsh et par Lh

Pour la majorité de ces gènes, la localisation de leur expression est prédite dans les cellules somatiques, ce qui est cohérent avec la présence des récepteurs sur les cellules somatiques testiculaires. Beaucoup de ces gènes se caractérisent aussi par une forte expression au stade VIII ainsi que par une régulation positive par les androgènes *in vivo* (Rolland *et al.* 2009; Rolland *et al.* 2013). Cela suggère que les régulations mises en évidence *in vitro* à partir d'explants testiculaires à des stades précoces prennent place *in vivo* aux dernières phases du cycle naturel de reproduction, caractérisées par des taux plasmatiques très élevés des 2 gonadotropines et des stéroïdes (T, 11KT, DHP). Dans cette catégorie de gènes, nous retrouvons des gènes impliqués dans la maturation et l'excrétion du sperme, avec des fonctions dans la composition du fluide séminal et l'hydratation du sperme (*slc26a4*, *vt1*, *timp2*, *ca6*, *ca5a*). Le gène *nanos3* appartient également à cette catégorie de gènes et pourrait être impliqué dans l'arrêt du cycle car chez la souris, la surexpression de Nanos3 empêcherait la différenciation des spermatogonies (Lolicato *et al.* 2008). Nous retrouvons aussi des gènes codant pour des acteurs clés de la stéroïdogenèse (*star*, *hsd3b1*, *cyp17a1*, *cyp11b2-2*) ce qui est cohérent avec la présence des 2 types de récepteurs sur les cellules de Leydig et l'action stimulatrice sur la synthèse de stéroïdes par les 2 hormones chez les poissons. Par contre, les androgènes ont une action régulatrice négative sur ces derniers gènes. Ce rétrocontrôle négatif

est sans doute nécessaire pour contrôler finement la production locale de stéroïdes au sein du testicule.

La méta-analyse réalisée à partir des données d'expression de gènes obtenues dans les 2 expériences d'incubation *in vitro* de tissu testiculaire, en présence de Fsh et Lh d'une part, et de Fsh seule ou combinée au trilostane d'autre part, a permis de mettre en lumière qu'une grande partie de l'action commune de Fsh et Lh est probablement relayée par la production de stéroïdes biologiquement actifs (**IV**). Cette conclusion est cohérente avec l'activité stimulatrice de Fsh et de Lh sur la production de stéroïdes dans la gonade.

L'usage du trilostane apporte une réponse globale mais ne permet pas d'identifier quels stéroïdes sont impliqués dans la régulation des gènes. L'inhibition de l'enzyme 3β-HSD par le trilostane peut affecter la synthèse de tous les stéroïdes de la voie delta 4 que ce soit les progestagènes, les androgènes et les estrogènes. Parmi ces stéroïdes, l'implication des androgènes dans la régulation de la spermatogénèse semblait très probable en début de cycle chez les poissons. Pour vérifier leur action sur l'expression des gènes *in vitro* et la comparer à celle de la Fsh, nous avons effectué, avec le même pool d'explants testiculaires, des incubations en présence de Fsh et d'androgènes aromatisables (T, MT) et non aromatisables (11KT). Seul un faible nombre de gènes a répondu significativement au traitement par les androgènes. Ce résultat est surprenant comparé à celui obtenu *in vivo* chez la truite, où un apport exogène d'androgènes (T et 11KT) induit la dérégulation de plusieurs centaines de gènes (Rolland *et al.* 2013). La réponse *in vitro* de quelques gènes pris comme témoins positifs s'est révélée plutôt faible ce qui ne nous permet pas d'exclure un problème technique lié à notre dispositif expérimental. Une autre explication possible est que des stéroïdes autres que les androgènes, en particulier la DHP, seraient des régulateurs plus efficaces de l'expression des gènes. Chez l'anguille (Miura *et al.* 2006) et le poisson zèbre (Chen *et al.* 2013), la DHP joue un rôle dans les étapes précoces de la spermatogenèse en stimulant la prolifération et la différenciation des spermatogonies et en favorisant l'entrée en méiose des cellules germinales. D'autres approches expérimentales peuvent être envisagées pour étudier l'action primaire d'un stéroïde en particulier sur l'expression des gènes ; il peut s'agir de réaliser des incubations d'explants testiculaires en présence de différents stéroïdes (E2, T, DHP) ou au contraire, en présence d'antagonistes non stéroïdiens des récepteurs aux stéroïdes (par exemple, la flutamide pour les AR, clomifène ou tamoxifène pour les ER, la mifépristone pour les PR).

De façon intéressante, quand on considère l'action de la Fsh relayée par les stéroïdes, la situation chez les poissons n'est pas aussi opposée qu'on pourrait le croire à celle décrite chez les mammifères, et plus précisément les rongeurs. En effet chez les rongeurs, bien que la LH soit le seul facteur de régulation directe de la stéroïdogenèse, il a été montré que FSH pouvait aussi, de façon indirecte, réguler les fonctions des cellules de Leydig (Vihko *et al.* 1991; Matikainen *et al.* 1994). La FSH et les androgènes sont essentiels à une spermatogenèse normale et leurs rôles respectifs dans ce processus ont été précisés grâce à des modèles de souris déficientes en gonadotropines et/ou délétées du récepteur de FSH ou des androgènes (Abel *et al.* 2008; O'Shaughnessy *et al.* 2009; O'Shaughnessy *et al.* 2010). Ces études concluent que la FSH est nécessaire pour optimiser le nombre de cellules germinales dans les premières étapes de la spermatogenèse et que les androgènes de leur côté sont déterminants pour l'achèvement de la méiose. L'action de la FSH sur le transcriptome testiculaire a aussi été étudiée (McLean *et al.* 2002; Sadate-Ngatchou *et al.* 2004; Abel *et al.* 2009). Chez les souris *hpg*, dépourvues de gonadotropines, un traitement à moyen terme par la FSH *in vivo*, modifie l'expression d'un grand nombre de transcrits, non seulement dans les cellules de Sertoli, mais aussi dans les cellules de Leydig et dans une moindre mesure dans les cellules germinales (Abel *et al.* 2009). Ainsi la FSH semble capable de réguler indirectement l'activité des cellules de Leydig en augmentant l'expression de transcrits spécifiques. En retour, les androgènes, sécrétés par les cellules de Leydig, contrôleraient les fonctions du tube séminifère, en partie à travers la régulation de gènes impliqués dans le métabolisme de la vitamine A, le cytosquelette et l'homéostasie du fluide tubulaire (O'Shaughnessy *et al.* 2007). Par ailleurs, les androgènes et la FSH peuvent exercer des régulations redondantes ou synergiques sur certains gènes des cellules de Sertoli codant des facteurs essentiels pour la progression des cellules germinales (Abel *et al.* 2008). Une telle redondance d'action entre Fsh et les androgènes pourrait peut-être expliquer que la réponse à Fsh de plusieurs gènes est réduite mais pas supprimée en présence de trilostane.

Ainsi donc, au moins chez la souris, le mécanisme d'action de la FSH peut impliquer ou requérir la voie des stéroïdes. Chez les poissons, la principale différence réside dans l'action directe de la Fsh sur les cellules de Leydig pour stimuler la production d'androgènes. Malgré un mode différent du contrôle de leur production, les androgènes régulent les fonctions tubulaires, chez la truite, par des processus biologiques similaires à ceux des mammifères (Schulz *et al.* 2010; Rolland *et al.* 2013). Par exemple, la communication cellule somatique - cellule germinale par le biais de la matrice interstitielle et de la membrane basale est

essentielle pour la spermatogenèse chez les mammifères (Cheng *et al.* 2010). Parmi les gènes régulés par l'intermédiaire des stéroïdes, nous trouvons plusieurs gènes codant pour des composants du cytosquelette et de la matrice extracellulaire parmi lesquels, la smootheline, la béta-spectrine, la filamin-binding LIM protéine 1, la dystrophine, des métalloprotéinases et leur inhibiteur. Nous trouvons aussi le gène codant pour claudine 11, protéine qui joue un rôle majeur dans les jonctions serrées spécifiques de l'espace intercellulaire.

b. Gènes préférentiellement régulés par Fsh

Chez les poissons, on sait encore peu de choses des actions de la Fsh sur les fonctions testiculaires autres que sur la stéroïdogénèse. Nos données constituent donc une avancée majeure car pour la première fois nous avons pu montrer que 1- la Fsh régule l'expression testiculaire de nombreux gènes et 2- une part importante de cette action spécifique de Fsh s'exerce indépendamment de la production des stéroïdes.

Sur la base des données obtenues précédemment dans notre équipe, on peut prédire, pour une forte proportion des gènes, une expression localisée dans les cellules somatiques (Rolland *et al.* 2009). De plus, des données obtenues chez les rongeurs nous permettent d'attribuer une origine sertolienne à plusieurs gènes trouvés ici régulés par Fsh indépendamment des stéroïdes (McLean *et al.* 2002; Chalmel *et al.* 2007).

Le trilostane n'inhibant pas la voie de synthèse delta 5, on ne peut écarter une action de ces stéroïdes sur l'expression des gènes. Toutefois, chez les poissons, la voie de synthèse delta 4 est majoritaire (Fostier *et al.* 1987) et les stéroïdes biologiquement actifs sont essentiellement issus de cette voie de synthèse (T, E2, DHP). De plus, parmi les gènes de cette catégorie, seul un très faible nombre présente une différence d'expression significative entre le groupe contrôle et le groupe traité au trilostane seul. Cette donnée accrédite l'idée d'une absence d'action des stéroïdes de la voie delta5.

c. Gènes préférentiellement régulés par Lh

Le nombre de gènes détectés comme préférentiellement régulés par Lh est 2 fois plus grand que celui des gènes préférentiellement régulés par Fsh. Cela pourrait être lié à la proportion d'hormone réellement biologiquement active contenue dans chacune des

préparations qui fait que, à quantité égale de protéines purifiées, l'activité biologique est différente. Cependant, la réponse des explants testiculaires à la stimulation par Fsh et par Lh s'est révélée de même ampleur en termes de production de 11KT, après 48 et 96 heures d'incubation.

L'existence même de ce groupe de gènes exclusivement régulés par Lh pose question. En effet, on postule généralement que la Lh agit essentiellement via la production des stéroïdes et on s'attendrait, chez la truite, à ce que la Fsh régule aussi ces gènes. Il pourrait donc s'agir de gènes liés à la fonction stéroïdogène mais en amont de celle-ci, ou de gènes liés à des fonctions des cellules de Leydig autres que la stéroïdogénèse et que la Fsh ne régule pas. Un approfondissement des modes d'action de Lh nécessiterait de déterminer les gènes dont la réponse à Lh est dépendante ou non de la production de stéroïdes, comme nous l'avons fait pour les gènes régulés par la Fsh.

La liste des gènes issue des premières analyses ne permet pas de repérer des voies de régulation connues qui seraient pertinentes dans le contrôle de la spermatogenèse. Fait intéressant, une forte proportion de ces gènes (39.5 % des gènes Up et 53 % des gènes Down) serait exprimée dans les cellules germinales, ce qui irait bien avec une action présumée de la Lh sur la méiose et la spermiogénèse via les androgènes. Mais de façon surprenante, nous n'avons pas détecté de gènes connus pour la spermatogénèse.

Dans la littérature, il existe peu d'étude à grande échelle de l'action de Lh sur l'expression des gènes qui permettrait de faire une comparaison avec nos données. Des travaux publiés en 2010 par Zhou et collaborateurs ont identifié chez le rat, de façon indirecte, des gènes somatiques régulés par LH (Zhou *et al.* 2010). Parmi les gènes surexprimés, on trouve environ 60 gènes impliqués dans les métabolismes lipidique et stéroïdien. La comparaison avec nos données chez la truite montre que ces gènes correspondent davantage à ceux qui sont co-régulés par Lh et Fsh, qu'à ceux qui sont régulés par Lh uniquement. Cela conforte le fait que chez les téléostéens, la Fsh est, comme la Lh, un régulateur majeur de la stéroïdogenèse.

V. Quelles conclusions peut-on tirer sur le rôle de Fsh ?

L'ensemble de nos données permettent d'émettre des hypothèses fortes mais qui restent à vérifier, sur le rôle de Fsh sur les fonctions testiculaires au cours du cycle de reproduction. Un intérêt particulier est porté sur le début de cycle, période qui inclut la phase de prolifération et de différenciation des spermatogonies et l'entrée en méiose, mais nous évoquerons aussi la fin du cycle notamment la phase de maturation et de l'excrétion du sperme.

1. Rôles de Fsh en début de cycle :

La détection de Fshr dès le stade I de développement testiculaire (immature) de la truite est compatible avec le rôle attribué à la Fsh dans les étapes précoces de la spermatogenèse, quand Fsh est la seule gonadotropine sécrétée (Figure 10A) (Gomez *et al.* 1999). En particulier, Fsh a été impliquée dans la prolifération précoce des cellules de Sertoli (Schulz *et al.* 2003) et stimule *in vitro* la prolifération des spermatogonies A (Loir 1999). Chez le bar, le traitement *in vivo* par de la Fsh recombinante accélère la maturation gonadique et le développement spermatogénétique (Mazon *et al.* 2011). Notre étude du transcriptome a mis en évidence des effets stimulateurs et des effets inhibiteurs de Fsh sur l'expression de gènes codant pour des facteurs de la famille des TGF béta (*amh*, *inhba*, *fstl3*), pour des facteurs de croissance (*igf1b*, *mdka*) ou encore pour des composants de la matrice extracellulaire et du cytosquelette, facteurs qui constituent des médiateurs probables de l'action de la Fsh sur la spermatogénèse.

- ✓ *Régulation de la voie TGF beta*

Amh

L'AMH, sécrétée dans la gonade génétiquement mâle au moment de la différenciation sexuelle chez les mammifères, provoque une régression rapide des canaux de Müller. Bien que chez les poissons, il n'y ait pas de canaux de Müller, l'Amh est produite dans le testicule, dans les cellules de Sertoli (Rolland *et al.* 2009; Skaar *et al.* 2011). Chez la truite, son expression est régulée au cours de la gamétogenèse, avec une expression maximale au stade I de la spermatogenèse qui décroit de façon progressive dès le début des proliférations actives des et de la différenciation des cellules germinales (Figure 14) (Rolland *et al.* 2009). Nous avons montré que le transcrit *amh* est régulé négativement par Fsh et cette régulation ne dépend pas de la production de stéroïdes induite par Fsh. L'Amh empêche la prolifération et

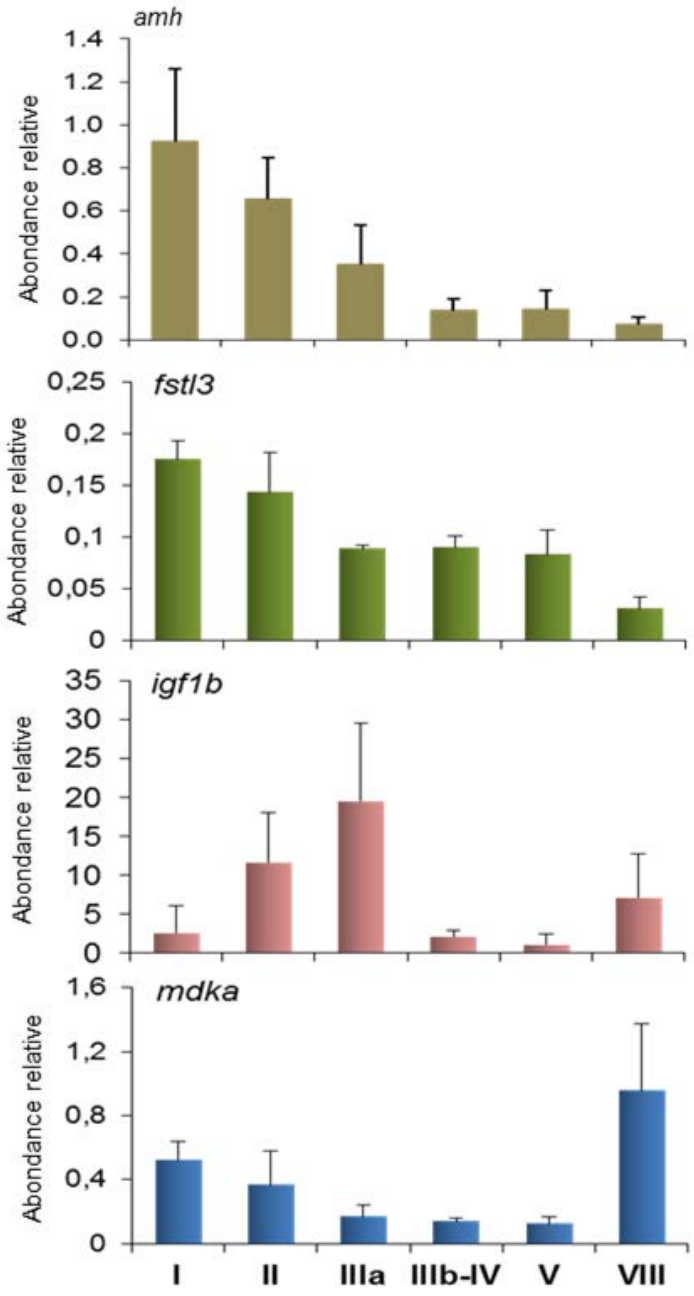

Figure 14 : Abondance relative des ARNm de Amh, Fstl3, Igf1b et Mdka mesurés par qPCR dans du tissu testiculaire à différents stades de la maturation spermatogénétique.

la différenciation des spermatogonies (Miura *et al.* 2002) et inhibe la production de stéroïdes induite par Fsh (Skaar *et al.* 2011). Le rôle des androgènes sur la régulation de l'Amh est contradictoire selon les espèces étudiées : *in vitro*, le taux d'accumulation du transcrit *amh* est négativement régulé par la 11KT chez l'anguille alors que chez la truite et le poisson zèbre, les androgènes n'ont pas d'effet significatif sur le niveau des transcrits. *In vivo*, la supplémentation en androgènes exogènes (T, 11KT et OHA) entraine une diminution du niveau des transcrits au bout de 7 jours, chez la truite (Rolland et al., 2013). Cet effet observé *in vivo* pourrait être indirect, dû au rétrocontrôle positif par la testostérone sur la synthèse et la sécrétion de Fsh chez la truite immature (Magri *et al.* 1985; Breton *et al.* 1997).

Fstl3 et activine (inhba)

La follistatine-like 3 est une glycoprotéine exprimée dans le testicule et capable d'inhiber des membres de la famille TGFβ et plus spécifiquement l'activine, modulant ou réduisant ainsi cette voie de signalisation très importante pour le bon déroulement de la spermatogenèse. Chez la souris, Fstl3 est localisée dans les cellules de Leydig, les spermatogonies et les spermatides (Xia *et al.* 2004). Une étude récente chez la souris vient de démontrer que cette glycoprotéine intervient à la fois dans le contrôle de la taille du testicule, en régulant le nombre de cellules de Sertoli, et dans la régression testiculaire liée à l'âge (Oldknow *et al.* 2013). Son rôle chez la truite reste hypothétique mais on peut supposer que Fstl3 antagonise aussi les effets de l'activine. De son côté, l'activine A est connue pour promouvoir la prolifération des cellules de Sertoli et des gonocytes dans le testicule fœtal et post-natal des rongeurs et empêche la différenciation des gonocytes en spermatogonies (Meehan *et al.* 2000). Par contre, une fois la différenciation des spermatogonies engagée, l'activine favorise la prolifération de ces spermatogonies (Mather *et al.* 1990).

Le profil d'expression de *fstl3*, déterminé chez la truite mâle au cours du cycle de maturation gonadique (Figure 14), révèle que son expression est la plus forte en début de cycle (stades I et II) et la plus faible en fin de cycle (stade VIII). Nous avons montré que Fsh a une action stimulatrice sur l'expression de *fstl3* et *inha* et une action inhibitrice sur l'expression du transcrit *inhba*. En début de développement spermatogénétique, ces 2 effets pourraient concourir à réduire la voie de l'activine et ainsi favoriser la différenciation des spermatogonies.

- ✓ *Régulation de facteurs de croissance*

La voie Igf

L'Igf1b est un paralogue de l'Igf1 de mammifère identifié récemment chez les téléostéens et dont l'expression est dominante dans les gonades (Wang *et al.* 2008; Zou *et al.* 2009). Chez la truite, seule l'expression d'*igf1b* (pas celle d'*igf1a* ni d'*igf2*, les 2 autres formes présentes) est fortement régulée par Fsh et dans une moindre mesure par Lh. Chez le poisson zèbre, il a aussi été montré que l'hCG augmentait l'expression d'*igf1b* dans l'ovaire (Irwin *et al.* 2012).

Des travaux plus anciens menés dans notre laboratoire sur la truite arc-en-ciel, ont montré que les récepteurs Igf sont présents sur les spermatogonies et que l'IGF-1 stimule leur prolifération *in vitro* (Loir *et al.* 1994; Le Gac *et al.* 1996). En outre, la Fsh stimule la prolifération des spermatogonies en co-culture avec des cellules de Sertoli (Loir 1999). Enfin, chez le poisson zèbre, l'expression du transcrit *igf1b* a été localisée dans les cellules de Sertoli entourant les spermatogonies A indifférenciées (Schulz et al., données non publiées). Ces observations suggèrent que Fsh augmente la production d'Igf1b par les cellules de Sertoli et à son tour, Igf1b pourrait agir comme un médiateur direct de la prolifération des spermatogonies.

D'autres données chez le tilapia décrivent l'expression du transcrit *igf1b* dans les cellules interstitielles (Wang *et al.* 2008). Bien qu'en contradiction avec la situation décrite chez le poisson zèbre, la localisation d'*igf1b* dans les cellules de Leydig est comparable à celle d'IGF1 chez les mammifères (Lin *et al.* 1990). Chez le rat, l'IGF1 produite par les cellules de Leydig participe à la régulation de la stéroïdogenèse, de façon autocrine (Lin *et al.* 1986). Cela pourrait aussi être le cas chez les poissons puisque chez le tilapia, il a été démontré que l'Igf1b recombinant régulait les transcrits codant pour les enzymes de la stéroïdogenèse et les facteurs de transcription (*dmrt1*, *nr5a1*, *foxl2*) impliqués dans leur régulation (Li *et al.* 2012). Les auteurs concluent qu'Igf1b pourrait être le facteur de croissance primaire impliqué dans la régulation de la stéroïdogénèse gonadique.

Les transcrits de l'*igf1b* sont détectables à tous les stades étudiés et leur abondance relative augmente fortement mais de façon transitoire aux stades II et IIIa, correspondant à la prolifération rapide des spermatogonies (Figure 14). Leur niveau d'expression tend à

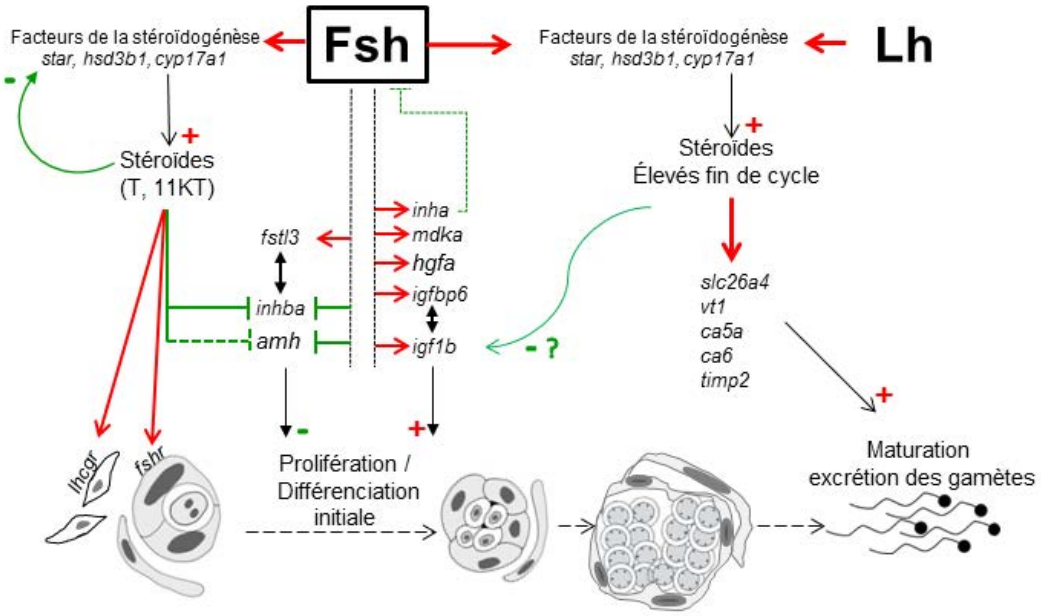

Figure 15 : Schéma récapitulant les médiateurs moléculaires probables de l'action de la Fsh sur les étapes précoces et tardives du cycle de développement spermatogénétique chez la truite. Les flèches rouges indiquent un effet stimulateur tandis que les lignes vertes indiquent un effet inhibiteur sur les gènes. Les flèches noires représentent l'implication potentielle des gènes dans les différents processus, proposée sur la base de leur fonction déjà connue chez les mammifères ou les poissons et sur la base de leur profil d'expression au cours du cycle.

réaugmenter tardivement au stade de spermiation. Ce profil d'expression de l'Igf1b chez la truite est cohérent avec des actions paracrines et autocrines à la fois sur la spermatogenèse et sur la stéroïdogenèse. Enfin, on remarque que l'abondance relative du transcrit *igf1b* baisse rapidement en deuxième partie de cycle (figure 14) alors que la Fsh et son récepteur augmentent. Cette évolution traduit peut-être un effet inhibiteur des fortes concentrations circulantes d'androgènes, hypothèse fondée sur l'effet antagoniste des androgènes sur *igf1b* décrit dans l'article **IV** (et figure 13).

En plus d'*igf1b*, nous avons noté que Fsh stimule l'expression de la transcription d'*igfbp6*. Les protéines de liaison des Igf (Igfbp) sont des protéines de transport extracellulaires, considérées comme des modulateurs importants de l'action des Igfs. La régulation forte d'*igf1b* et *igfbp6* par Fsh renforce l'idée que la voie de régulation de l'Igf est une voie paracrine majeure qui relaie les actions de la Fsh sur la spermatogenèse et sur la stéroïdogénèse.

Mdka

Ce gène, stimulé spécifiquement par Fsh, code une midkine, protéine de la famille des heparin-binding growth factor. La midkine peut réguler des activités telles que la prolifération et la migration cellulaires et est impliquée dans plusieurs processus de développement comme la neurogenèse ou l'angiogenèse. Son rôle dans les fonctions testiculaires est inconnu. Une étude i*n vitro* a montré que la midkine augmente l'activité de prolifération des cellules germinales primordiales ovariennes, les maintenant dans un état moins différencié via une inhibition de l'expression de Dazl (Shen *et al.* 2012). Chez la truite, son expression décroit régulièrement entre les stades I et V et augmente à nouveau au stade VIII (Figure 14). Ce gène représente un nouveau candidat dont la fonction mérite d'être approfondie et que nous n'aurions pas pu identifier si nous n'avions pas mis en œuvre l'approche sans *a priori*.

A partir de l'ensemble de ces données, nous pouvons déduire que l'action première de Fsh pourrait être d'augmenter la réceptivité des cellules de Sertoli et vraisemblablement aussi des cellules de Leydig à sa propre action ce qui rendrait la gonade apte à produire les stéroïdes et les facteurs paracrines nécessaires à la progression de la spermatogénèse. Fsh agirait sur la prolifération et la différenciation des spermatogonies d'une part, en supprimant l'action inhibitrice de l'Amh et de l'activine et d'autre part en stimulant l'expression de l'Igf1b et de

Mdka qui elles, favoriseraient la prolifération des spermatogonies. Ces effets se conjuguent pour permettre l'engagement des cellules germinales dans la spermatogenèse. Les 4 gènes décrits ci-dessus présentent 4 profils d'expression différents, ce qui suggère que des facteurs autres que la Fsh régulent aussi leur expression de manière dynamique.

2. Rôles de Fsh et de Lh en fin de cycle :

En fin de cycle, on observe une forte élévation des transcrits *fshr* et *lhcgr*, coïncidant avec les plus forts niveaux circulants de Fsh et de Lh. La forte élévation des transcrits *lhcgr* en fin de cycle est cohérente avec le rôle prépondérant attribué à la signalisation Lh/Lhcgr dans le contrôle des phases finales du cycle de reproduction, en particulier, la maturation et l'excrétion des gamètes (Weil *et al.* 1983; Takashima *et al.* 1984; Prat *et al.* 1996). Une étude fine au cours de la maturation finale chez la truite mâle montre que l'élévation brutale des transcrits *lhcgr* coïncide avec le plus fort pourcentage de mâles spermiants et avec le volume maximal de sperme émis (Figure 10B, d'après données non publiées de Bellaiche et al.). Ces données concernant l'accumulation des transcrits *lhcgr* sont également cohérentes avec celles obtenues antérieurement au laboratoire qui démontraient chez la truite mâle une forte augmentation des sites de liaison à Lh (sGtH) au cours de la période de spermiation (Le Gac *et al.* 1987). Le taux de transcrits *lhcgr* semble donc bien corrélé avec les quantités de protéines produites.

Le fait que *fshr* ait un profil d'expression similaire à *lhcgr* en fin de cycle pose la question du rôle de Fsh/Fshr au cours des étapes tardives de la gamétogenèse. Le signal Fsh/Fshr pourrait participer au contrôle de la maturation finale, en régulant des gènes impliqués dans les processus permettant la formation d'un fluide séminal adapté à la maturation des spermatozoïdes et à leur transit vers le spermiducte. Nos données concernant les gènes régulés par les gonadotropines viennent soutenir cette hypothèse : i) parmi les gènes régulés par Fsh on trouve des gènes impliqués dans les échanges ioniques, la régulation du pH et l'hydratation ii) ces gènes sont fortement exprimés au stade VIII et iii) ces gènes sont aussi positivement régulés par un traitement *in vivo* aux androgènes. Il est intéressant de noter que chez la truite femelle, une élévation de l'expression du transcrit *fshr* est associée à une forte compétence du follicule à entrer en maturation (Bobe *et al.* 2003) suggérant là aussi un rôle de Fshr dans le contrôle de la fin de la gamétogenèse.

Nos données montrent que la Fsh stimule des gènes impliqués dans la biosynthèse des stéroïdes (*star*, *hsd3b1*). Ainsi l'augmentation de la Fsh circulante et de ses récepteurs

pourrait contribuer avec la Lh, à la forte augmentation de la production de nombreux stéroïdes sexuels incluant les androgènes et les progestagènes. Chez les salmonidés, immédiatement avant ou pendant la période d'émission du sperme, il y a une modification de la stéroïdogenèse qui se traduit par une augmentation la production de DHP au détriment de la production d'androgènes (Sakai *et al.* 1989). Il a été observé que l'action de la Lh sur la production de DHP augmente en fin de cycle alors que celle de la Fsh diminue (Le Gac *et al.* 1987) (Planas *et al.* 1995). La réponse du testicule à l'action de Fsh sur la production de 11KT reste inchangée à cette même phase du cycle (Planas *et al.* 1995). L'augmentation de Fsh en fin de cycle, suivie d'une baisse au cours de la spermiation pourrait participer à la mise en place du shift androgènes ➔ DHP. La Lh serait plus impliquée dans la production de la DHP connue pour ses effets inducteurs de la maturation finale : hydratation du sperme, amélioration de la motilité des spermatozoïdes par altération du pH et de la fluidité du liquide séminal (pour revue (Scott *et al.* 2010).

PERSPECTIVES

I. Préciser le profil d'expression des récepteurs des gonadotropines pour mieux définir les cibles cellulaires directes de la Fsh chez la truite

✓ En priorité, nous souhaiterions localiser les récepteurs chez la truite : les travaux préliminaires visant à localiser les transcrits des récepteurs des gonadotropines dans notre modèle ne nous ont pas permis de conclure. Or, cette donnée nous paraît indispensable pour démontrer quelles sont les cellules testiculaires qui sont les cibles de l'action directe de chacune des gonadotropines et pour conforter notre hypothèse d'une action directe de Fsh sur les cellules de Leydig pour stimuler la production de stéroïdes, en absence de Lh circulante. Un moyen d'obtenir des résultats plus concluants qu'avec l'HIS pourrait être la microdissection laser. Cette technique permettrait d'isoler à partir de tissu testiculaire et sous contrôle morphologique, les cellules ou groupes de cellules du compartiment tubulaire de celles du compartiment interstitiel et contribuerait à augmenter la sensibilité et la spécificité des analyses ultérieures par qPCR de la quantité d'ARNm.

✓ A moyen terme, il parait judicieux de préciser leur expression à d'autres périodes charnières du cycle de vie

Nos données ainsi que les données de la littérature indiquent que Fsh est impliquée dans la prolifération des spermatogonies, mais nous ne savons pas si Fsh régule aussi les proliférations lentes des précurseurs ou spermatogonies indifférenciées. Un début de réponse pourrait être apporté en suivant l'expression de son récepteur à des étapes encore plus précoces du développement testiculaire que celles que nous avons décrites.

A l'opposé, la forte élévation des transcrits de Fshr en fin de cycle soulève des questions encore non élucidées sur le rôle du signal Fsh/Fshr dans la maturation des gamètes et/ou dans la préparation du cycle suivant. Il serait intéressant d'étudier la période de transition entre la fin du premier cycle et le début du second cycle de reproduction et de comparer les données avec celles observées lors de la mise en place de la première vague spermatogénétique.

II. Action de la Fsh dans le contrôle de la prolifération /différenciation des spermatogonies

1. Etude des réponses cellulaires à Fsh

L'étude des mécanismes d'action de la Fsh sur la spermatogénèse pourrait être approfondie par l'étude *in vitro* de l'action cellulaire de Fsh pour rechercher des effets mitotiques et/ou anti-apoptotiques de Fsh sur les cellules de Sertoli et/ou les cellules germinales et faire le lien fonctionnel avec les gènes régulés par Fsh. Alors que Loir (Loir 1999) avait pu montrer que Fsh stimule la prolifération des cellules germinales isolées en culture, l'étude *in vitro* des évènements cellulaires en réponse à l'action de Fsh sur explants testiculaires a fait l'objet de plusieurs tentatives peu fructueuses dans notre équipe y compris de façon préliminaire par moi-même au cours de cette thèse. Nous avons incubé, à long terme, des explants testiculaires en présence ou non de Fsh et avons utilisé un anticorps anti phospho-histone H3 pour marquer les cellules en division. Ce travail n'a pas permis d'observer d'effet significatif de Fsh sur la prolifération des cellules germinales. Nous suspectons l'existence de verrous méthodologiques ou liés à notre modèle, qui empêcheraient de détecter des évènements quantitativement faibles. C'est pourquoi, nous étudierons en parallèle les effets des protéines codées par les gènes régulés par Fsh.

2. Etude de la fonctionnalité des gènes régulés par Fsh

Les données accumulées dans le cadre de cette thèse améliorent notre compréhension des régulations moléculaires des fonctions testiculaires par les gonadotropines chez les poissons. Elles nous fournissent plusieurs dizaines de nouveaux candidats qui pourraient relayer l'action de Fsh sur le déclenchement de la spermatogénèse active (transition vers la phase de multiplication rapide et de différenciation méiotique), et/ou sur la maturation des gamètes. L'une des suites immédiates à donner à notre analyse de la régulation hormonale du transcriptome testiculaire est de se focaliser sur la fonction des gènes régulés par Fsh et susceptibles d'agir sur la prolifération et/ou la différenciation des cellules germinales. Nos analyses devront combiner les informations obtenues sur les régulations hormonales, le profil d'expression spatio-temporelle et l'impact biologique de ces gènes sur la prolifération/différenciation des spermatogonies. Il faudra donc dans un premier temps préciser plusieurs points importants:

- préciser les profils d'expression temporelle des candidats au cours du développement testiculaire, comme nous avons commencé à le faire pour *amh*, *igf1b*, *fstl3* et *mdka*.

- déterminer la localisation cellulaire des gènes Fsh dépendants. Cette localisation sera déterminée par hybridation *in situ* des transcrits sur des coupes de testicules à différents stades de la maturation gonadique.
- pour les gènes régulés par la Fsh via la production de stéroïdes (avec un effet agoniste ou antagoniste) identifier les stéroïdes impliqués. Des échantillons obtenus après incubation *in vitro* en présence de 11KT, MT, E2 et DHP à deux concentrations différentes sont en cours d'étude.

En parallèle, l'équipe se donnera les moyens d'évaluer l'impact, la signification ou l'importance biologique des gènes candidats par des approches *in vitro* reposant sur l'exposition des cellules germinales ou d'explants testiculaires à des protéines recombinantes produites par des cellules somatiques. L'effet de ces protéines sur la prolifération ou l'apoptose des cellules germinales sera étudié. Il sera aussi possible de mettre en œuvre des approches fonctionnelles *in vivo* par inactivation des gènes candidats dans des lignées transgéniques (Talen, CRISPR /Cas9 actuellement testées au laboratoire). L'objectif premier de l'équipe étant de préciser les évènements qui président au déclenchement de la spermatogenèse « active » à la puberté, les critères suivants seront appliqués pour sélectionner un nombre restreint de candidats:

- ✓ régulation par Fsh
- ✓ profil d'expression cohérent avec un rôle précoce dans la prolifération / différenciation des cellules germinales
- ✓ expression dans les cellules somatiques
- ✓ fonction se rapportant à des facteurs de croissance /cytokines d'intérêt pour un processus de développement comme la spermatogenèse (fonction prédite sur la base de la GeneOntology et de la bibliographie, le plus souvent mammalienne)

3. Identification de nouvelles voies de signalisation activées par le signal Fsh

Le choix d'utiliser un même support (microarray 9K à ADNc de truite) pour les expériences successives sur le transcriptome testiculaire a été fait pour faciliter et enrichir l'interprétation des données acquises (voir plus haut). Elle a cependant limité l'identification de médiateurs parmi les 6000 gènes non redondants, représentant seulement 10% des gènes exprimés. Certes, l'utilisation de cette puce nous a permis d'identifier un nombre non négligeable de candidats. Cependant les nouvelles générations de puces et les nouvelles

techniques de séquençage à haut débit, de type RNAseq d'Illumina, offriront également l'opportunité d'approfondir l'exploitation des échantillons collectés lors de la thèse en se rapprochant d'une étude exhaustive du transcriptome et la possibilité de travailler les « pathways » complexes impliqués dans la réponse à Fsh. Le séquençage récent du génome de truite devrait faciliter l'interprétation des résultats.

REFERENCES

Abdennebi L, Couture L, Grebert D, Pajot E, Salesse R & Remy JJ 1999 Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. Journal of Molecular Endocrinology 22 151-159.

Abdennebi L, Chun EY, Jammes H, Wei D & Remy JJ 2003 Maintenance of sexual immaturity in male mice and bucks by immunization against N-terminal peptides of the follicle-stimulating hormone receptor. Biology of Reproduction 68 323-327.

Abel MH, Baker PJ, Charlton HM, Monteiro A, Verhoeven G, De GK, Guillou F & O'Shaughnessy PJ 2008 Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. Endocrinology 149 3279-3285.

Abel MH, Baban D, Lee S, Charlton HM & O'Shaughnessy PJ 2009 Effects of FSH on testicular mRNA transcript levels in the hypogonadal mouse. Journal of Molecular Endocrinology 42 291-303.

Aizen J, Kowalsman N, Kobayashi M, Hollander L, Sohn YC, Yoshizaki G, Niv MY & Levavi-Sivan B 2012 Experimental and computational study of inter- and intra- species specificity of gonadotropins for various gonadotropin receptors. Mol. Cell Endocrinol. 364 89-100.

Alam MA, Kobayashi Y, Hirai T & Nakamura M 2010 Isolation, characterization and expression analyses of FSH receptor in protogynous grouper. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 156 364-371.

Andersson E, Nijenhuis W, Male R, Swanson P, Bogerd J, Taranger GL & Schulz RW 2009 Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. Gen. Comp Endocrinol. 163 329-339.

Baker PJ, Pakarinen P, Huhtaniemi IT, Abel MH, Charlton HM, Kumar TR & O'Shaughnessy PJ 2003 Failure of normal Leydig cell development in follicle-stimulating hormone (FSH) receptor-deficient mice, but not FSHbeta-deficient mice: role for constitutive FSH receptor activity. Endocrinology 144 138-145.

Basu D & Bhattacharya S 2002 Purification of two types of gonadotropin receptors from carp ovarian follicles: overlapping recognition by two different ligands. General and Comparative Endocrinology 129 152-162.

Baynes SM & Scott AP 1985 Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). Gen. Comp Endocrinol. 57 150-160.

Billard R & Fontaine M 1969 Hypophysectomie et spermatogenese chez *Poecilia reticulata* (Poisson Cyprinodontidae). Comptes rendus de l'Académie d'Agriculture de France 1856-1859.

Billard R, Burzawa-Gerard E & Breton B 1970 Regeneration de la spermatogenese du Cyprin hypophysectomise *Carassius auratus* par un facteur gonadotrope hautement purifie de carpe. Comptes Rendus de l'Académie des Sciences. Paris 271 1896-1899.

Billard R 1979 La gametogenese, le cycle sexuel et le controle de la reproduction chez les poissons teleosteens. Bulletin français de pisciculture 273 117-136.

Billard R, Fostier A, Weil C & Breton B 1982 Endocrine Control of Spermatogenesis in Teleost Fish. Canadian Journal of Fisheries and Aquatic Sciences 39 65-79.

Billard R, Marcel J & de Montalembert G 1983 Stimulation de la spermiation chez le brochet (*Esox lucius*). In Le Brochet : Gestion Dans Le Milieu Naturel Et Elevage, pp 109-132. R Billard Paris: INRA

Bobe J, Maugars G, Nguyen T, Rime H & Jalabert B 2003 Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. Molecular reproduction and development. 66 46-53.

Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ & Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biology of Reproduction 64 1633-1643.

Borg B 1994 Androgens in teleost fishes. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 109 219-245.

Breton B, Le Gac F & Sambroni E 1986 Gonadotropin hormone (GtH) receptors in the ovary of the brown trout *Salmo trutta* L. in vitro studies. General and Comparative Endocrinology 64 163-171.

Breton B & Sambroni E 1989 Evolution du nombre des récepteurs gonadotropes ovariens au cours du cycle reproducteur annuel chez la truite fario *Salmo trutta* L. Comptes rendus de l'académie des sciences. Série III: sciences de la vie 308 495-500.

Breton B, Mikolajczyk T, Popek W, Bieniarz K & Epler P 1991 Neuropeptide Y stimulates in vivo gonadotropin secretion in teleost fish. Gen. Comp Endocrinol. 84 277-283.

Breton B, Mykolajczyk T & Popek W 1993 The neuroendocrine control of the gonadotropin (GTH2) secretion in teleost fish. In Aquaculture : Fundamental and Applied Research, pp 199-215. Eds B Lahlou & P Vitiello Washington: American Geophysical Union

Breton B & Sambroni E 1996 Steroid activation of the brain-pituitary complex gonadotropic function in the triploid rainbow trout *Oncorhynchus mykiss*. Gen. Comp Endocrinol. 101 155-164.

Breton B, Sambroni E, Govoroun M & Weil C 1997 Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. Comptes rendus de l'académie des sciences. Série III: sciences de la vie 320 783-789.

Breton B, Govoroun M & Mikolajczyk T 1998 GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: Relationship with pituitary responsiveness to GnRH-A stimulation. General and Comparative Endocrinology 111 38-50.

Carreau S, Bouraima-Lelong H & Delalande C 2012 Estrogen, a female hormone involved in spermatogenesis. Adv. Med. Sci. 57 31-36.

Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, Gattiker A, Moore J, Patard JJ, Wolgemuth DJ & Jegou B 2007 The conserved transcriptome in human and rodent male gametogenesis. Proc Natl Acad Sci USA 104 8346-8351.

Chauvigne F, Tingaud-Sequeira A, Agulleiro MJ, Calusinska M, Gomez A, Finn RN & Cerda J 2010 Functional and evolutionary analysis of flatfish gonadotropin receptors reveals cladal- and lineage-level divergence of the teleost glycoprotein receptor family. Biol Reprod. 82 1088-1102.

Chauvigne F, Verdura S, Mazon MJ, Duncan N, Zanuy S, Gomez A & Cerda J 2012 Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. Biol Reprod. 87 35.

Chaves-Pozo E, Liarte S, Vargas-Chacoff L, Garcia-Lopez A, Mulero V, Meseguer J, Mancera JM & Garcia-Ayala A 2007 17Beta-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. Biol. Reprod. 76 142-148.

Chen SX, Bogerd J, Schoonen NE, Martijn J, de Waal PP & Schulz RW 2013 A progestin (17alpha,20beta-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. Gen. Comp Endocrinol. 185 1-9.

Cheng CY, Wong EWP, Yan HHN & Mruk DD 2010 Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: New insights and advances. Molecular and Cellular Endocrinology 315 49-56.

Couture L, Naharisoa H, Grebert D, Remy JJ, Pajot-Augy E, Bozon V, Haertle T & Salesse R 1996 Peptide and immunochemical mapping of the ectodomain of the porcine LH receptor. J. Mol. Endocrinol. 16 15-25.

Crepieux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, Guillou F & Reiter E 2001 The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene 20 4696-4709.

Dattatreyamurty B, Figgs LW & Reichert LE, Jr. 1987 Physical and functional association of follitropin receptors with cholera toxin-sensitive guanine nucleotide-binding protein. Journal of Biological Chemistry 262 11737-11745.

Dickey JT & Swanson P 1998 Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). J. Mol. Endocrinol. 21 291-306.

Dunlap KD & Zakon HH 1998 Behavioral actions of androgens and androgen receptor expression in the electrocommunication system of an electric fish, *Eigenmannia virescens*. Horm. Behav. 34 30-38.

Evans HM & Long JA 1922 Characteristic Effects upon Growth, Oestrus and Ovulation Induced by the Intraperitoneal Administration of Fresh Anterior Hypophyseal Substance. Proc. Natl. Acad. Sci. U. S. A 8 38-39.

Fevold HL, Hisaw FL & Leonard SL 1931 The gonad-stimulating and the luteinizing hormones of the anterior lobe of th hypophysis. Am. J. Physiol. 97 291-301.

Fostier A, Billard R, Breton B, Legendre M & Marlot S 1982 Plasma 11-Oxotestosterone and Gonadotropin During the Beginning of Spermiation in Rainbow-Trout (*Salmo-Gairdneri R*). General and Comparative Endocrinology 46 428-434.

Fostier A, Le Gac F & Loir M 1987 Steroids in male reproduction. In Reproductive Physiology of Fish, pp 239-245. Eds DR IdlerLW CrimJM Walsh St-John's: University of Newfounland (CA)

Frazier AL, Robbins LS, Stork PJ, Sprengel R, Segaloff DL & Cone RD 1990 Isolation of TSH and LH/CG receptor cDNAs from human thyroid: regulation by tissue specific splicing. Mol. Endocrinol. 4 1264-1276.

Garcia-Lopez A, Bogerd J, Granneman JC, van DW, Trant JM, Taranger GL & Schulz RW 2009 Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150 357-365.

Garcia-Lopez A, de Jonge H, Nobrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J & Schulz RW 2010 Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. Endocrinology 151 2349-2360.

Gautier A, Le Gac F & Lareyre JJ 2011 The gsdf gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes. Gene 472 7-17.

Gloaguen P, Crepieux P, Heitzler D, Poupon A & Reiter E 2011 Mapping the follicle-stimulating hormone-induced signaling networks. Front Endocrinol. (Lausanne) 2 45.

Gomez JM, Weil C, Ollitrault M, Le Bail PY & Le Gac F 1999 Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp Endocrinol. 113 413-428.

Govoroun M, Chyb J & Breton B 1998 Immunological cross-reactivity between rainbow trout GTH I and GTH II and their alpha and beta subunits: Application to the development of specific radioimmunoassays. General and Comparative Endocrinology 111 28-37.

Govoroun MS, Huet JC, Pernollet JC & Breton B 1997 Use of immobilized metal ion affinity chromatography and dye-ligand chromatography for the separation and purification of rainbow trout pituitary gonadotropins, GTH I and GTH II. Journal of Chromatography B 698 35-46.

Grier HJ, Linton JR, Leatherland JF & De Vlaming VL 1980 Structural evidence for two different testicular types in teleost fishes. Am. J. Anat. 159 331-345.

Gudermann T, Birnbaumer M & Birnbaumer L 1992 Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca2+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. Journal of Biological Chemistry 267 4479-4488.

Heppell J & Davis HL 2000 Application of DNA vaccine technology to aquaculture. Adv. Drug Deliv. Rev. 43 29-43.

Hewitt LF 1929 Hormones of the anterior pituitary lobe. Biochem. J. 23 718-725.

Hirai T, Oba Y & Nagahama Y 2002 Fish gonadotropin receptors: molecular characterization and expression during gametogenesis. Fisheries Science 68 675-678.

Hoar WS 1965 Comparative Physiology: Hormones and Reproduction in Fishes. Annual Review of Physiology 27 51-70.

Hoar WS 1969 Reproduction. In Fish Physiology, pp 1-72. Eds WS Hoar & DJ Randall New York: Academic Press

Holdcraft RW & Braun RE 2004 Hormonal regulation of spermatogenesis. Int. J. Androl 27 335-342.

Huhtaniemi I 2006 Mutations along the pituitary-gonadal axis affecting sexual maturation: Novel information from transgenic and knockout mice. Molecular and Cellular Endocrinology 254-255 84-90.

Irwin DA & Van Der Kraak G 2012 Regulation and actions of insulin-like growth factors in the ovary of zebrafish (*Danio rerio*). General and Comparative Endocrinology 177 187-194.

Jeng SR, Yueh WS, Chen GR, Lee YH, Dufour S & Chang CF 2007 Differential expression and regulation of gonadotropins and their receptors in the Japanese eel, *Anguilla japonica*. General and Comparative Endocrinology 154 161-173.

Kagawa H, Tanaka H, Okuzawa K & Kobayashi M 1998 GTH II but Not GTH I Induces Final Maturation and the Development of Maturational Competence of Oocytes of Red Seabream in Vitro. General and Comparative Endocrinology 112 80-88.

Kah O, Lethimonier C, Somoza G, Guilgur LG, Vaillant C & Lareyre JJ 2007 GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen. Comp Endocrinol. 153 346-364.

Kajimura S, Yoshiura Y, Suzuki M & Aida K 2001 cDNA cloning of two gonadotropin beta subunits (GTH-Ibeta and -IIbeta) and their expression profiles during gametogenesis in the Japanese flounder (*Paralichthys olivaceus*). Gen. Comp Endocrinol. 122 117-129.

Kamei H, Kaneko T & Aida K 2006 In vivo gonadotropic effects of recombinant Japanese eel follicle-stimulating hormone. Aquaculture 261 771-775.

Kanamori A, Kagawa H & Nagahama Y 1987 Gonadotropin receptors in the postovulatory ovary of amago salmon (*Oncorhynchus rhodurus*). General and Comparative Endocrinology 66 210-217.

Kazeto Y, Kohara M, Miura T, Miura C, Yamaguchi S, Trant JM, Adachi S & Yamauchi K 2008 Japanese eel follicle-stimulating hormone (fsh) and luteinizing hormone (lh): production of biologically active recombinant fsh and lh by Drosophila s2 cells and their differential actions on the reproductive biology. Biology of Reproduction 79 938-946.

Kerr JB & Sharpe RM 1985 Follicle-stimulating hormone induction of Leydig cell maturation. Endocrinology 116 2592-2604.

Kobayashi T & Andersen O 2008 The gonadotropin receptors FSH-R and LH-R of Atlantic halibut (Hippoglossus hippoglossus), 1: isolation of multiple transcripts encoding full-length and truncated variants of FSH-R. Gen. Comp Endocrinol. 156 584-594.

Kumakura N, Okuzawa K, Gen K, Yamaguchi S, Lim BS & Kagawa H 2004 Effects of gonadotropin-releasing hormone on pituitary-ovarian axis of one-year old pre-pubertal red seabream. Gen. Comp Endocrinol. 138 105-112.

Kumar RS & Trant JM 2001a Piscine glycoprotein hormone (gonadotropin and thyrotropin) receptors: a review of recent developments. Comp Biochem. Physiol B Biochem. Mol. Biol. 129 347-355.

Kumar RS, Ijiri S & Trant JM 2001b Molecular biology of the channel catfish gonadotropin receptors: 2. Complementary DNA cloning, functional expression, and seasonal gene expression of the follicle-stimulating hormone receptor. Biology of Reproduction 65 710-717.

Kumar RS, Ijiri S & Trant JM 2001c Molecular biology of channel catfish gonadotropin receptors: 1. Cloning of a functional luteinizing hormone receptor and preovulatory induction of gene expression. Biology of Reproduction 64 1010-1018.

Kumar TR 2005 What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? Reproduction 130 293-302.

Kumar TR 2007 Functional analysis of LH[beta] knockout mice. Molecular and Cellular Endocrinology 269 81-84.

Kusakabe M, Nakamura I, Evans J, Swanson P & Young G 2006 Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. Journal of Endocrinology 189 541-554.

Kwok HF, So WK, Wang Y & Ge W 2005 Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors--evidence for their distinct functions in follicle development. Biology of Reproduction 72 1370-1381.

Laan M, Richmond H, He C & Campbell RK 2002 Zebrafish as a model for vertebrate reproduction: characterization of the first functional zebrafish (*Danio rerio*) gonadotropin receptor. General and Comparative Endocrinology 125 349-364.

Le Gac F & Fostier A 1987 Evolution of sensitivity to GtH and GtH receptors in the trout testis (*Salmo gairdneri*). Proceedings of the third international symposium on the Reproductive Physiology of Fish 73.

Le Gac F, Breton B & Bougoussa M 1988 Gonadotropic hormone (GtH) receptors in the testis of the trout *Salmo gairdnerii*: in vitro studies. Fish Physiology and Biochemistry 5 209-217.

Le Gac F, Loir M, Le Bail PY & Ollitrault M 1996 Insulin-like growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. Mol Reprod Dev 44 23-35.

Legendre M & Jalabert B 1988 Physiologie de la reproduction. In Biologie Et Ecologie Des Poissons D'Eau Douce Africains, pp 153-187. Eds C LevequeMN BrutonGW Ssentongo Paris:

Lepa A, Siwicki AK & Terech-Majewska E 2010 Application of DNA vaccines in fish. Pol. J. Vet. Sci. 13 213-215.

Levavi-Sivan B, Bogerd J, Mananos EL, Gomez A & Lareyre JJ 2010 Perspectives on fish gonadotropins and their receptors. General and Comparative Endocrinology 165 412-437.

Li M, Wu F, Gu Y, Wang T, Wang H, Yang S, Sun Y, Zhou L, Huang X, Jiao B, Cheng CH & Wang D 2012 Insulin-Like Growth Factor 3 Regulates Expression of Genes Encoding Steroidogenic Enzymes and Key Transcription Factors in the Nile Tilapia Gonad. Biology of Reproduction 86 163.

Lin T, Haskell J, Vinson N & Terracio L 1986 Direct stimulatory effects of insulin-like growth factor-I on Leydig cell steroidogenesis in primary culture. Biochem. Biophys. Res. Commun. 137 950-956.

Lin T, Wang DL, Calkins JH, Guo H, Chi R & Housley PR 1990 Regulation of insulin-like growth factor-I messenger ribonucleic acid expression in Leydig cells. Mol. Cell Endocrinol. 73 147-152.

Lin XW & Peter RE 1996 Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. Gen. Comp Endocrinol. 101 282-296.

Loir M & Le Gac F 1994 Insulin-like growth factor-I and -II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes. Biology of Reproduction 51 1154-1163.

Loir M 1999 Spermatogonia of rainbow trout: II. in vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. Molecular reproduction and development. 53 434-442.

Lolicato F, Marino R, Paronetto MP, Pellegrini M, Dolci S, Geremia R & Grimaldi P 2008 Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population. Developmental Biology 313 725-738.

Loosfelt H, Misrahi M, Atger M, Salesse R, Vu Hai-Luu Thi MT, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J & . 1989 Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain. Science 245 525-528.

Ma X, Dong Y, Matzuk MM & Kumar TR 2004 Targeted disruption of luteinizing hormone {beta}-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. Proceedings of the National Academy of Sciences 101 17294-17299.

Magri MH, Solari A, Billard R & Reinaud P 1985 Influence of testosterone on precocious sexual development in immature rainbow trout. Gen. Comp Endocrinol. 57 411-421.

Mananos EL, Anglade I, Chyb J, Saligaut C, Breton B & Kah O 1999 Involvement of gamma-aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. Neuroendocrinology 69 269-280.

Mather JP, Attie KM, Woodruff TK, Rice GC & Phillips DM 1990 Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology 127 3206-3214.

Matikainen T, Toppari J, Vihko KK & Huhtaniemi I 1994 Effects of recombinant human FSH in immature hypophysectomized male rats: evidence for Leydig cell-mediated action on spermatogenesis. Journal of Endocrinology 141 449-457.

Maugars G & Schmitz M 2008 Expression of gonadotropin and gonadotropin receptor genes during early sexual maturation in male Atlantic salmon parr. Mol Reprod Dev. 75 403-413.

Maugars G & Schmitz M 2006 Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (*Salmo salar* L.). General and Comparative Endocrinology 149 108-117.

Mazon MJ, Zanuy S, Carillo M & Gomez A 2011 Use of somatic gene transfer for studying gonadotropin actions on spermatogenesis in european sea bass (*Dicentrarchus labrax*). Indian J. Sci. Technol. , 4 143-144.

McFarland KC, Sprengel R, Phillips HS, Kohler M, Rosemblit N, Nikolics K, Segaloff DL & Seeburg PH 1989 Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 245 494-499.

McLean DJ, Friel PJ, Pouchnik D & Griswold MD 2002 Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. Mol. Endocrinol. 16 2780-2792.

Meachem SJ, Ruwanpura SM, Ziolkowski J, Ague JM, Skinner MK & Loveland KL 2005 Developmentally distinct in vivo effects of FSH on proliferation and apoptosis during testis maturation. Journal of Endocrinology 186 429-446.

Means AR, MacDougall E, Soderling TR & Corbin JD 1974 Testicular adenosine 3':5'-monophosphate-dependent protein kinase. Regulation by follicle-stimulating hormone. Journal of Biological Chemistry 249 1231-1238.

Meehan T, Schlatt S, O'Bryan MK, de Kretser DM & Loveland KL 2000 Regulation of Germ Cell and Sertoli Cell Development by Activin, Follistatin, and FSH. Developmental Biology 220 225-237.

Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O & Pakdel F 2002 Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66 1881-1892.

Minegishi T, Nakamura K, Takakura Y, Miyamoto K, Hasegawa Y, Ibuki Y, Igarashi M & Minegish T 1990 Cloning and sequencing of human LH/hCG receptor cDNA. Biochem. Biophys. Res. Commun. 172 1049-1054.

Minegishi T, Nakamura K, Takakura Y, Ibuki Y, Igarashi M & Minegish T 1991 Cloning and sequencing of human FSH receptor cDNA. Biochem. Biophys. Res. Commun. 175 1125-1130.

Miura T, Yamauchi K, Takahashi H & Nagahama Y 1991 Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc. Natl Acad Sci U. S A 88 5774-5778.

Miura T, Yamauchi K, Takahashi H & Nagahama Y 1992 The role of hormones in the acquisition of sperm motility in salmonid fish. J. Exp. Zool. 261 359-363.

Miura T, Miura C, Ohta T, Nader MR, Todo T & Yamauchi K 1999 Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res. Commun. 264 230-234.

Miura T, Miura C, Konda Y & Yamauchi K 2002 Spermatogenesis-preventing substance in Japanese eel. Development 129 2689-2697.

Miura T, Higuchi M, Ozaki Y, Ohta T & Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proc. Natl Acad Sci U. S A 103 7333-7338.

Miwa S, Yan L & Swanson P 1994 Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. Biology of Reproduction 50 629-642.

Montserrat N, Gonzalez A, Mendez E, Piferrer F & Planas JV 2004 Effects of follicle stimulating hormone on estradiol-17[beta] production and P-450 aromatase (CYP19) activity and mRNA expression in brown trout vitellogenic ovarian follicles in vitro. General and Comparative Endocrinology 137 123-131.

Musnier A, Leon K, Morales J, Reiter E, Boulo T, Costache V, Vourc'h P, Heitzler D, Oulhen N, Poupon A, Boulben S, Cormier P & Crepieux P 2012 mRNA-selective translation induced by FSH in primary Sertoli cells. Mol. Endocrinol. 26 669-680.

Mylonas CC, Scott AP & Zohar Y 1997 Plasma gonadotropin II, sex steroids, and thyroid hormones in wild striped bass (*Morone saxatilis*) during spermiation and final oocyte maturation. Gen. Comp Endocrinol. 108 223-236.

Nagahama Y 1994 Endocrine regulation of gametogenesis in fish. International Journal of Developmental Biology 38 217-229.

Nagahama Y 1997 17 alpha,20 beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. Steroids 62 190-196.

Nozaki M, Naito N, Swanson P, Dickhoff WW, Nakai Y, Suzuki K & Kawauchi H 1990a Salmonid pituitary gonadotrophs. II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). Gen. Comp Endocrinol. 77 358-367.

Nozaki M, Naito N, Swanson P, Miyata K, Nakai Y, Oota Y, Suzuki K & Kawauchi H 1990b Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. Gen. Comp Endocrinol. 77 348-357.

O'Donnell L, Robertson KM, Jones ME & Simpson ER 2001 Estrogen and spermatogenesis. Endocr. Rev. 22 289-318.

O'Shaughnessy PJ, Abel M, Charlton HM, Hu B, Johnston H & Baker PJ 2007 Altered expression of genes involved in regulation of vitamin A metabolism, solute transportation, and cytoskeletal function in the androgen-insensitive tfm mouse testis. Endocrinology 148 2914-2924.

O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ & Abel MH 2009 Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: Data from mutant and genetically modified mice. Molecular and Cellular Endocrinology 306 2-8.

O'Shaughnessy PJ, Verhoeven G, De GK, Monteiro A & Abel MH 2010 Direct action through the sertoli cells is essential for androgen stimulation of spermatogenesis. Endocrinology 151 2343-2348.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H & Nagahama Y 1999a The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochemical biophysical research communications 265 366-371.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H & Nagahama Y 1999b Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochemical biophysical research communications 263 584-590.

Oba Y, Hirai T, Yoshiura Y, Kobayashi T & Nagahama Y 2001 Fish gonadotropin and thyrotropin receptors: the evolution of glycoprotein hormone receptors in vertebrates. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129 441-448.

Ohta T, Miyake H, Miura C, Kamei H, Aida K & Miura T 2007 Follicle-Stimulating Hormone Induces Spermatogenesis Mediated by Androgen Production in Japanese Eel, *Anguilla japonica*. Biology of Reproduction 77 970-977.

Oldknow KJ, Seebacher J, Goswami T, Villen J, Pitsillides AA, O'Shaughnessy PJ, Gygi SP, Schneyer AL & Mukherjee A 2013 Follistatin-like 3 (FSTL3) mediated silencing of transforming growth factor beta (TGFbeta) signaling is essential for testicular aging and regulating testis size. Endocrinology 154 1310-1320.

Parenti LR & Grier HJ 2004 Evolution and Phylogeny of Gonad Morphology in Bony Fishes. Integrative and Comparative Biology 44 333-348.

Pickford GE & Atz JW 1957 In The Physiology of the Pituitary Gland of Fishes New York Zoological Society (New York)

Pierce JG & Parsons TF 1981 Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50 465-495.

Planas JV & Swanson P 1995 Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. Biology of Reproduction 52 697-704.

Planas JV, Athos J, Goetz FW & Swanson P 2000 Regulation of Ovarian Steroidogenesis In Vitro by Follicle-Stimulating Hormone and Luteinizing Hormone During Sexual Maturation in Salmonid Fish. Biology of Reproduction 62 1262-1269.

Prat F, Sumpter JP & Tyler CR 1996 Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction 54 1375-1382.

Pudney J 1995 Spermatogenesis in nonmammalian vertebrates. Microscopy Research and Technique 32 459-497.

Querat B, Jutisz M, Fontaine YA & Counis R 1990 Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone alpha-subunit of the European eel. Mol. Cell Endocrinol. 71 253-259.

Radu A, Pichon C, Camparo P, Antoine M, Allory Y, Couvelard A, Fromont G, Hai MT & Ghinea N 2010 Expression of follicle-stimulating hormone receptor in tumor blood vessels. N. Engl. J. Med. 363 1621-1630.

Rao AJ, Ramachandra SG, Ramesh V, Couture L, Abdennebi L, Salesse R & Remy JJ 2004 Induction of infertility in adult male bonnet monkeys by immunization with phage-expressed peptides of the extracellular domain of FSH receptor. Reproductive biomedicine online 8 385-391.

Rao CV & Lei ZM 2007 The past, present and future of nongonadal LH/hCG actions in reproductive biology and medicine. Mol. Cell Endocrinol. 269 2-8.

Remy JJ, Lahbib-Mansais Y, Yerle M, Bozon V, Couture L, Pajot E, Greber D & Salesse R 1995 The porcine follitropin receptor: cDNA cloning, functional expression and chromosomal localization of the gene. Gene 163 257-261.

Remy JJ, Couture L, Rabesona H, Haertle T & Salesse R 1996 Immunization against exon 1 decapeptides from the lutropin/choriogonadotropin receptor or the follitropin receptor as potential male contraceptive. Journal of Reproductive Immunology 32 37-54.

Rocha A, Gomez A, Zanuy S, Cerda-Reverter JM & Carrillo M 2007 Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and functional activity. Mol. Cell Endocrinol. 272 63-76.

Rolland AD, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ, Esquerre D, Hugot K, Houlgatte R, Chalmel F & Le Gac F 2009 Expression profiling of rainbow trout testis

development identifies evolutionary conserved genes involved in spermatogenesis. BMC Genomics 10 546.

Rolland AD, Lardenois A, Goupil AS, Lareyre JJ, Houlgatte R, Chalmel F & Le Gac F 2013 Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. PLoS. One. 8 e53302.

Sadate-Ngatchou PI, Pouchnik DJ & Griswold MD 2004 Follicle-stimulating hormone induced changes in gene expression of murine testis. Mol. Endocrinol. 18 2805-2816.

Saha A, Sambroni E, Bogerd J, Schulz RW, Le Gac F & Lareyre JJ 2011 The cell context influences rainbow trout gonadotropin receptors selectivity. Indian J. Sci. Technol. , 4 45-46.

Sakai N, Ueda H, Suzuki N & Nagahama Y 1989 Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. Gen. Comp Endocrinol. 75 231-240.

Salesse R & Garnier J 1996 Glycoprotein hormone receptors: new molecular supports for old biological functions., pp 387-462. AG Lee JAI Press Inc

Saligaut C, Linard B, Mananos EL, Kah O, Breton B & Govoroun M 1998 Release of pituitary gonadotrophins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*): modulation by estradiol and catecholamines. General and Comparative Endocrinology 109 302-309.

Salmon C, Kagawa H, Adachi S, Nagahama Y & Fontaine YA 1984 Demonstration of specific gonadotropin binding sites of chum salmon (*Oncorhynchus keta*) in membrane preparations of granulosa cells from the ovary of amago salmon (*Oncorhynchus rhodurus*). Comptes rendus de l'académie des sciences. Série III: sciences de la vie 298 337-340.

Sambroni E, Le Gac F, Breton B & Lareyre JJ 2007 Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. J Endocrinol 195 213-228.

Sambroni E, Abdennebi-Najar L, Remy JJ & Le Gac F 2009 Delayed sexual maturation through gonadotropin receptor vaccination in the rainbow trout *Oncorhynchus mykiss*. General and Comparative Endocrinology 164 107-116.

Sambroni E, Rolland AD, Goupil AS, Lareyre JJ & Le Gac F 2011 Pathways used by androgens or FSH to regulate testis maturation. Indian J. Sci. Technol. , 4 141-142.

Sambroni E, Rolland AD, Lareyre JJ & Le Gac F 2013a Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis. J. Mol. Endocrinol. 50 1-18.

Sambroni E, Lareyre JJ & Le Gac F 2013b Fsh Controls Gene Expression in Fish both Independently of and through Steroid Mediation. PLoS ONE 8 e76684.

Schulz R, Dijk W & Bogerd J 2003 Sertoli cell proliferation and FSH signalling in African catfish, *Clarias gariepinus*. Fish Physiology and Biochemistry 28 223.

Schulz RW, Liemburg M, Garcia-Lopez A, Dijk W & Bogerd J 2008 Androgens modulate testicular androgen production in African catfish (*Clarias gariepinus*) depending on the stage of maturity and type of androgen. Gen. Comp Endocrinol. 156 154-163.

Schulz RW, de Franca LR, Lareyre JJ, LeGac F, Chiarini-Garcia H, Nobrega RH & Miura T 2010 Spermatogenesis in fish. General and Comparative Endocrinology 165 390-411.

Schulz RW, Menting S, Bogerd J, Franca LR, Vilela DAR & Godinho HP 2005 Sertoli Cell Proliferation in the Adult Testis-Evidence from Two Fish Species Belonging to Different Orders. Biology of Reproduction 73 891-898.

Scott AP, Sumpter JP & Stacey N 2010 The role of the maturation-inducing steroid, 17,20beta-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish. Biol. 76 183-224.

Sekine S, Saito A, Itoh H, Kawauchi H & Itoh S 1989 Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. Proceedings of the National Academy of Sciences 86 8645-8649.

Shen W, Park BW, Toms D & Li J 2012 Midkine promotes proliferation of primordial germ cells by inhibiting the expression of the deleted in azoospermia-like gene. Endocrinology 153 3482-3492.

Simoni M, Gromoll J & Nieschlag E 1997 The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 18 739-773.

Siraj A, Desestret V, Antoine M, Fromont G, Huerre M, Sanson M, Camparo P, Pichon C, Planeix F, Gonin J, Radu A & Ghinea N 2013 Expression of follicle-stimulating hormone receptor by the vascular endothelium in tumor metastases. BMC. Cancer 13 246.

Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW & Male R 2011 Proteolytically Activated, Recombinant Anti-Mullerian Hormone Inhibits Androgen Secretion, Proliferation, and Differentiation of Spermatogonia in Adult Zebrafish Testis Organ Cultures. Endocrinology 152 3527-3540.

So WK, Kwok HF & Ge W 2005 Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits--their spatial-temporal expression patterns and receptor specificity. Biology of Reproduction 72 1382-1396.

Soga T, Sakuma Y & Parhar IS 1998 Testosterone differentially regulates expression of GnRH messenger RNAs in the terminal nerve, preoptic and midbrain of male tilapia. Brain Res. Mol. Brain Res. 60 13-20.

Sprengel R, Braun T, Nikolics K, Segaloff DL & Seeburg PH 1990 The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA. Mol. Endocrinol. 4 525-530.

Sun L, Peng Y, Sharrow AC, Iqbal J, Zhang Z, Papachristou DJ, Zaidi S, Zhu LL, Yaroslavskiy BB, Zhou H, Zallone A, Sairam MR, Kumar TR, Bo W, Braun J, Cardoso-Landa L, Schaffler MB, Moonga BS, Blair HC & Zaidi M 2006 FSH directly regulates bone mass. Cell 125 247-260.

Suzuki K, Kawauchi H & Nagahama Y 1988a Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. General and Comparative Endocrinology 71 292-301.

Suzuki K, Nagahama Y & Kawauchi H 1988b Steroidogenic activities of two distinct salmon gonadotropins. General and Comparative Endocrinology 71 452-458.

Suzuki K, Kanamori A, Nagahama Y & Kawauchi H 1988c Development of salmon GTH I and GTH II radioimmunoassays. General and Comparative Endocrinology 71 459-467.

Swanson P, Bernard M, Nozaki M, Suzuki K, Kawauchi H & Dickoff WW 1989 Gonadotropins I and II in juvenile coho salmon. Fish Physiology and Biochemistry 7 169-176.

Swanson P, Suzuki K, Kawauchi H & Dickhoff WW 1991 Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biology of Reproduction 44 29-38.

Takashima F, Weil C, Billard R, Crim LW & Fostier A 1984 Stimulation of Spermiation by Lhrh Analog in Carp. Bulletin of the Japanese Society of Scientific Fisheries 50 1323-1329.

Taranger GL, Carrillo M, Fontaine P, Zanuy S, Felip A, Weltzien FA, Dufour S, Karlsen Ï, Norberg B, Andersson E & Hansen T 2010 Control of puberty in farmed fish. General and Comparative Endocrinology 165 483-515.

Tyler CR, Sumpter JP, Kawauchi H & Swanson P 1991 Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. General and Comparative Endocrinology 84 291-299.

Tyler CR, Pottinger TG, Coward K, Prat F, Beresford N & Maddix S 1997 Salmonid follicle-stimulating hormone (GtH I) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. Biology of Reproduction 57 1238-1244.

Ueda H, Kambegawa A & Nagahama Y 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. Gen. Comp Endocrinol. 59 24-30.

Utoh T, Okamura A, Yamada Y, Tanaka S, Mikawa N, Akazawa A, Horie N & Oka HP 2004 Reproductive cycle in reared male common Japanese conger, *Conger myriaster*. Aquaculture 240 589-605.

Van Der Kraak G, Suzuki K, Peter RE, Itoh H & Kawauchi H 1992 Properties of common carp gonadotropin I and gonadotropin II. General and Comparative Endocrinology 85 217-229.

Vickers ED, Laberge F, Adams BA, Hara TJ & Sherwood NM 2004 Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, Coregonus clupeaformis. Biol. Reprod. 70 1136-1146.

Vihko KK, Lapolt PS, Nishimori K & Hsueh AJW 1991 Stimulatory Effects of Recombinant Follicle-Stimulating Hormone on Leydig Cell Function and Spermatogenesis in Immature Hypophysectomized Rats. Endocrinology 129 1926-1932.

Vilela DAR, Silva SGB, Peixoto MTD, Godinho HP & Franca LR 2003 Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus*) model. Fish Physiology and Biochemistry 28 187-190.

Vischer HF & Bogerd J 2003a Cloning and functional characterization of a gonadal luteinizing hormone receptor complementary DNA from the African catfish (*Clarias gariepinus*). Biology of Reproduction 68 262-271.

Vischer HF, Granneman JC, Linskens MH, Schulz RW & Bogerd J 2003b Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. Journal of Molecular Endocrinology 31 133-140.

Wang DS, Jiao B, Hu C, Huang X, Liu Z & Cheng CHK 2008 Discovery of a gonad-specific IGF subtype in teleost. Biochemical and Biophysical Research Communications 367 336-341.

Weil C & Crim LW 1983 Administration of Lhrh Analogs in Various Ways - Effect on the Advancement of Spermiation in Prespawning Landlocked Salmon, *Salmo-Salar*. Aquaculture 35 103-115.

Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S & Valotaire Y 1995 Preliminary evidence suggesting variations of GtH 1 and GtH2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp Endocrinol. 100 327-333.

Weltzien FA, Andersson E, Andersen O, Shalchian-Tabrizi K & Norberg B 2004 The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comp Biochem. Physiol A Mol. Integr. Physiol 137 447-477.

Wu C, Patino R, Davis KB & Chang X 2001 Localization of estrogen receptor alpha and beta RNA in germinal and nongerminal epithelia of the channel catfish testis. Gen. Comp Endocrinol. 124 12-20.

Xia Y, Sidis Y & Schneyer A 2004 Overexpression of Follistatin-Like 3 in Gonads Causes Defects in Gonadal Development and Function in Transgenic Mice. Molecular Endocrinology 18 979-994.

Yamazaki F & Donaldson EM 1968 The effects of partially purified salmon pituitary gonadotropin on spermatogenesis, vitellogenesis, and ovulation in hypophysectomid goldfish (*Carassius auratus*). Gen. Comp Endocrinol. 11 292-299.

Yan L, Swanson P & Dickhoff WW 1992 A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biology of Reproduction 47 418-427.

Yoshiura Y, Suetake H & Aida K 1999 Duality of gonadotropin in a primitive teleost, Japanese eel (*Anguilla japonica*). Gen. Comp Endocrinol. 114 121-131.

Zhang SB, Dattatreyamurty B & Reichert LE, Jr. 1991 Differential roles of high and low affinity guanosine 5'-triphosphate binding sites in the regulation of follicle-stimulating hormone binding to receptor and signal transduction in bovine calf testis membranes. Endocrinology 128 295-302.

Zhou W, Bolden-Tiller OU, Shetty G, Shao SH, Weng CC, Pakarinen P, Liu Z, Stivers DN & Meistrich ML 2010 Changes in Gene Expression in Somatic Cells of Rat Testes Resulting from Hormonal Modulation and Radiation-Induced Germ Cell Depletion. Biology of Reproduction 82 54-65.

Zhou X 2010 Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice. J. Androl 31 235-243.

Zhu Y, Rice CD, Pang Y, Pace M & Thomas P 2003 Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. U. S. A 100 2231-2236.

Zohar Y, Munoz-Cueto JA, Elizur A & Kah O 2010 Neuroendocrinology of reproduction in teleost fish. Gen. Comp Endocrinol. 165 438-455.

Zou S, Kamei H, Modi Z & Duan C 2009 Zebrafish IGF Genes: Gene Duplication, Conservation and Divergence, and Novel Roles in Midline and Notochord Development. PLoS ONE 4 e7026.

Résumé

Chez les vertébrés, le processus de la spermatogénèse est directement contrôlé par deux hormones gonadotropes hypophysaires, Fsh et Lh. Chez les salmonidés, les profils de sécrétion des 2 hormones diffèrent et présentent des variations significatives au cours du cycle de développement spermatogénétique, suggérant que Fsh et Lh interviennent à des étapes différentes du processus. A la différence des mammifères, chez les poissons les 2 gonadotropines exercent une forte activité stéroïdogène, et par ailleurs il a été rapporté par plusieurs auteurs que leurs récepteurs seraient moins sélectifs vis-à-vis des 2 ligands. Ainsi, le périmètre des actions respectives de Fsh et de Lh n'est pas défini chez les poissons. D'autre part, les mécanismes de l'action de Fsh qui ne seraient pas relayés par les stéroïdes sont très mal connus. Chez la truite, nous avons déterminé que chaque gonadotropine agit essentiellement par l'intermédiaire de son récepteur respectif. L'analyse des variations du transcriptome testiculaire après un traitement *in vitro* par les hormones de la reproduction (Fsh, Lh, androgènes) a permis 1- de révéler des actions distinctes de Fsh et de Lh sur l'expression des gènes, 2- de mettre en évidence deux mécanismes d'action de la Fsh, l'un dépendant et l'autre indépendant de la production de stéroïdes et 3- d'identifier plusieurs acteurs d'interaction cellulaire régulés par Fsh, et probablement impliqués dans les étapes précoces de prolifération ou de différenciation des cellules germinales, tels que l'hormone antimüllérienne, la midkine, l'insulin-like growth factor1b, la follistatine-like 3 et l'activine, 4-de proposer une implication de Fsh dans les évènements tardifs de maturation et d'excrétion du sperme. Au-delà des acquis concernant les régulations endocriniennes et moléculaires chez la truite, ces travaux constituent un apport de connaissances qui peut être étendu à d'autres téléostéens pour décrypter l'action propre à Fsh dans le déclenchement de la maturation pubertaire. Enfin, nous montrons qu'une vaccination contre les récepteurs des gonadotropines constitue une voie potentielle de contrôle des maturations précoces en élevage.

Abstract

In vertebrates, spermatogenesis is under the direct control of two pituitary gonadotropic hormones named Fsh and Lh. In salmonids, the 2 hormones are differentially secreted in the plasma through the reproductive cycle, suggesting that Fsh and Lh are involved in the regulation of different steps of the process. Unlike in mammals, in fish both gonadotropins exert a strong steroidogenic activity and, besides, some authors reported for their receptors a much lower selectivity towards the two ligands. Yet, their respective roles are not established in fish. Furthermore, the mechanisms of Fsh action that would not be mediated by steroids are poorly investigated. In trout, we showed that each gonadotropin mainly acts through its cognate receptor. The analysis of the variations of testicular transcriptome following an *in vitro* treatment with reproductive hormones (Fsh, Lh and androgens) permitted 1- to reveal that Fsh and Lh have distinct effects on gene expression, 2- to highlight two mechanisms of Fsh action, one dependent on the steroid production and the second one independent of that production, 3- to identify several Fsh regulated factors involved in cellular interactions and particularly in the control of germ cell proliferation / differentiation (anti mullerian hormone, midkine, insulin-like growth factor 1b, follistatin-like 3 and activin), 4- to propose an involvement of Fsh in late events of sperm maturation and release. In addition to knowledge on endocrine and molecular regulations in trout, this work provides a fund of knowledge useful in other teleosts to decipher the action of Fsh in triggering puberty onset. Finally, we showed that an immunization against the gonadotropin receptors is a potential method to delay sexual maturation in farmed fish.